

Cells, FAQs and a new monthly journal

Cell biology is flourishing, in the liveliness of its community and in the breadth and connectivity of the science. Hence today's launch of *Nature Cell Biology*, complementing *Nature's* continuing enthusiasm for the subject.

The grand quest of cell biologists is to understand how cells function and communicate with each other, working together to form tissues, organs and the great variety of life. Fundamental aspects such as cell division, protein sorting, programmed cell death (apoptosis) and the organization of the cell nucleus are less and less studied in isolation. The roles of signalling molecules and complexes, likewise, are increasingly understood in the context of cross-talking networks rather than individual pathways. The pace of discovery is rapid, the need for different areas of expertise to be brought together ever greater: cell biology is both competitive and integrative. That provides fertile ground for a monthly *Nature* journal intended to serve all those working on cell biological problems.

Whenever a new *Nature* journal is announced, *Nature's* editorial staff are frequently asked several questions. For example, will the community welcome it, given existing specialist publications? Our publishers' positive answer to this is based on two considerations. First, the signs are already good for *Nature Cell Biology*. Responses by researchers from questionnaires and discussion groups in several countries were positive, advance subscriptions are at a healthy level and, according to Annette Thomas, *Nature Cell Biology's* editor, there is a stack of high-quality papers on its way. Second, the journal faces similar publishing challenges to other monthly *Nature* journals when they were launched; they have all prospered, occupying leading positions in their respective disciplines.

Another FAQ: does this mean that *Nature* is abandoning the discipline? The answer is, as always, emphatically no — *Nature's* policies are unchanged. Our editors will continue to strive to publish the most complete studies providing major conceptual breakthroughs, not least in cell biology. The description of the enzyme (DNase) responsible for the breakdown of DNA during apoptosis (*Nature* 391, 43–50;

1998) is one celebrated example. A more recent set of papers exemplifies the virtues of multidisciplinary scope: four papers in our 8 April issue (*Nature* 398, 513–529; 1999) tell the tale of how a major signalling pathway (the Notch pathway) known to regulate cell-fate decisions during development depends on the actions of presenilin-1, a protein involved in processing the amyloid precursor protein implicated in Alzheimer's disease. And this week's issue sees an important step in the understanding of processes in the nucleus regulating the cell cycle (pages 818–823; News and Views, pages 757–758).

From today there are six monthly *Nature* journals (see <http://www.nature.com/author/natureguide.html>). *Nature Cell Biology* is no different from the others in seeking to publish landmark papers within its discipline, as well as authoritative comment. For a full discussion of its scope and ambitions, see the editorial in its first issue, available at <http://cellbio.nature.com>. Moreover, the journal has the same relationship to *Nature* itself. The editorial teams act fully independently: *Nature Cell Biology's* editor is the final arbiter of what it should publish, and there is no discussion between the journals before either takes a decision on a paper. But if *Nature* editors decide to reject a paper, for example on the grounds of insufficient breadth of impact, but feel that it would still be of exceptional interest to the cell biology community, they will suggest submission to *Nature Cell Biology* and offer to pass on all referees' comments to save time. This approach has proved successful with the other monthlies and, for authors, ensures that papers are judged on consistently independent criteria.

We look forward to serving the cell biology community with both journals, as it enjoys an era of unprecedented growth and diversity. □

Philip Campbell — Editor, *Nature*

Smallpox preservation advisable

Previous agreements notwithstanding, it makes sense not to destroy a key protective resource.

The decision by the Clinton administration not to proceed with the planned destruction of its store of smallpox (page 741) is well taken. A report last month by the US Institute of Medicine did not pronounce on whether the store should be destroyed, as the United States agreed to do in 1996 under a World Health Organization plan. But it lays out a persuasive case for the potential public-health benefits of retaining the live virus, which is frozen in a maximum-containment laboratory at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, accessible to only six highly trained scientists, and at a Russian government laboratory in Siberia.

The lack of effective antiviral medications would not be of so much concern were it not for the growing vulnerability of a population that has not been routinely vaccinated now for a generation. Were the disease to re-emerge — whether accidentally or at the hands of terrorists

or a rogue nation — the results could be catastrophic. Careful science under the auspices of the CDC, testing candidate medications against the virus in cell culture and, later, in monkey models, may offer the only serious weapon to respond to an epidemic, if not to prevent it.

In addition, a new, large population of the immunosuppressed now exists, thanks to organ transplantation, cancer chemotherapy and AIDS. These people deserve the potential benefit of a novel vaccine if the disease again became endemic. But development of such a vaccine would require the live virus.

Rightly, public-health advocates bemoan the prospect of any measure that increases the risk of a re-emergence of this scourge. But, given the impossibility of knowing who now possesses the virus, and from where it might appear, it is better to have a number of arrows in the quiver than to destroy the stock and cross our collective fingers. □



NIH plan brings global electronic journal a step nearer reality

[PARIS] Harold Varmus, director of the US National Institutes of Health (NIH), has drafted a formal proposal to create an unprecedented global electronic publishing venture covering all the biomedical sciences.

The move follows an initial proposal for a similar scheme — revealed earlier this year in *Nature* (397, 91; 1999) — by Patrick Brown, a researcher at the Howard Hughes Medical Institute at Stanford University School of Medicine, and David Lipman, director of the US National Council for Biotechnology Information (NCBI), which operates the PubMed and GenBank databases.

The draft proposal, “E-Biomed: a proposal for electronic publishing in the biomedical sciences”, was written by Varmus himself, with input from Brown and Lipman. It concludes that “The rise of the Internet offers an unprecedented opportunity to change scientific publishing in ways that could improve on virtually all aspects of the current system.”

The stated goal of the E-Biomed repository is to short-circuit the existing print journal system and centralize most of the world’s biomedical research publications at a single website. The repository would accept papers in every area of biomedicine, and provide free access to the full text of these to all readers.

Articles would either be posted without refereeing, or peer reviewed by third-party editorial boards. The whole would be run by an independent international governing body.



Varmus: keen to make E-Biomed a success.

Varmus emphasizes that a large shift to electronics could overcome many drawbacks of the existing publishing system, in particular the high costs of maintaining a plethora of high-price, low-circulation journals. In 1997, for example, the 121 member libraries of the US Association of Research Libraries spent US\$432 million of their \$2.4 billion total budget on journals, or about \$12,000 per scientist.

Fotis Kafatos, director of the European Molecular Biology Laboratory in Heidelberg, Germany, has argued that the proliferation of specialized journals has compartmentalized knowledge, and that electronics provides a means to organize information in ways more useful to readers.

The proposal similarly argues that taking advantage of the opportunities for organizing and disseminating knowledge offered by the Internet will depend on “low-cost, barrier-free access by scientists to all of the contributions of their fellow scientists in a conveniently displayed electronic format” (see *Nature* 397, 195–200; 1999).

Under Varmus’s proposal, the NIH, through the National Council for Biotech-

nology Information, would provide “financial, technical, and administrative” help to create an electronic archive — ‘E-Biomed’. The document emphasizes that NIH’s role should be to instigate the project, but that it would neither own nor operate the archive.

E-Biomed should be a community effort, according to the document, which suggests that it should be run by a governing board, made up of representatives of the “scientific community (readers and authors), editors, computer specialists, and funding agencies”.

Authors would retain their copyright, with papers being available freely from the archive. Articles would either be posted without refereeing — with immediate posting after “a simple screen for appropriateness,” or submitted with a request for peer review. This would be done by “editorial boards... identical to those that represent current print journals, or they might be composed of members of scientific societies or other groups approved by the E-Biomed governing board.”

Peer-reviewed articles would be posted immediately upon acceptance and, where passed by the editorial board, would be available both in the journal and in the archive.

Varmus also proposes that individual boards could create a new class of runner-up papers, which, while “less prestigious, still denote review and endorsement by the journal’s editorial board”. Commentaries would also be posted along with articles.

Varmus predicts that scientists would initially be reluctant to publish in E-Biomed, but would soon be won over “because of its simplicity, flexibility, and speed”.

The archive would also consider introducing open peer reviewing, where reviews would be published alongside articles and possibly signed. Another innovation proposed is that papers could be regularly updated, with the original article being preserved.

The report acknowledges that many outstanding questions remain. Although NIH has offered to provide start-up funding for the venture, it would require sustained financing. One possibility is that the archive should be publicly funded, says the report, adding that an alternative would be a system based on billing authors for page charges to publish, and then making the articles free.

Similarly, rules to cover the appointment, composition and authority of the governing board remain to be established. To address these questions, Varmus plans to circulate his proposal within the scientific community for “constructive comments... with the intention of putting a suitably revised plan into operation in the near future”. **Declan Butler**

National project to boost Japan’s net presence

[Tokyo] Full electronic versions of a range of Japanese scientific journals will soon be available, thanks to a government-led project giving technical support to help national research institutes and academic societies to deliver their publications via the Internet.

The online journal project was launched earlier this month as a collaboration between the Japan Science and Technology Corporation (JST) and the National Centre for Scientific Information Systems. It will allow journals to receive, review, edit and publish manuscripts electronically, at a fraction of the current cost.

Software developed by

the two organizations will enable authors to download templates for their articles which will be peer-reviewed and edited electronically. Authors, referees and editors will have real-time access to papers throughout the editorial process.

According to JST, the project has received ¥0.5 billion (US\$4.2 million) for the current fiscal year, and will initially do a test-run on the journals of the Physics Society of Japan. It plans to register at least 25 academic societies by next March.

A few Japanese scientific journals, such as the *Bulletin of the Chemical Society of Japan* and the *Journal of Biochemistry*, are already

available on the Internet. But Japan’s science publishers have been slow to use the advantages of Internet publishing.

“The demand for online journals has been very high, but academic societies, particularly the less powerful ones, did not have the capacity — either technical or financial — to venture into Internet publishing,” says Takashi Sahara of JST’s information business division. “Unlike international publishers such as Reed-Elsevier and Springer, which hold a very strong position in the online publishing market, Japanese publishers have not been able to take a lead in this area.” **Asako Saegusa**

Swiss reject GM trial to protect organics

[MUNICH] Switzerland's office for environment, forestry and agriculture (BUWAL) has rejected an application from AgrEvo, a German plant biotechnology company, to conduct field trials of its genetically modified (GM) herbicide-resistant 'T25' maize.

BUWAL officials say the site chosen by AgrEvo for its experiments was close to organic farms, and that cross-pollination leading to the contamination of organic crops could not be ruled out.

But the company says it is "mystified and upset" by the decision, and claims that it was made on "political" grounds. Less than a year ago a Swiss referendum rejected an initiative to ban such field trials by a two-thirds majority (see *Nature* 393, 507; 1998).

The application was one of the first two requests for field-trial licences since the early 1990s. The second, from the Swiss Institute, for field trials on a GM disease-resistant potato, was also rejected this month because of what were considered to be inadequate risk-assessment data. Technical data on the transgene were insufficient, said BUWAL, and the potato carried a marker gene for resistance to an antibiotic used in medicine.

In the case of the AgrEvo trials — to test characteristics of the resistance of T25 maize to the herbicide glufosinate — BUWAL says that the risk of contaminating organic crops is unacceptable. It argues that the application, initially made in 1997, did not include plans for monitoring potential gene flow to soil organisms or neighbouring plants.

"Swiss agriculture lives on the fact that its products are pure and natural," says BUWAL director Philippe Roch.

Wulff Hansen, a spokesman for the chemical company Plüss-Staufer, which represents AgrEvo in Switzerland, says the decision came out of the blue, as the company had already discussed cross-pollination and monitoring with BUWAL.

It had agreed, for example, to prevent the production of pollen by cutting the crop before it flowered. The nearest field used for organic farming was 300 metres away, says Hansen, and did not grow maize, the only type of crop where cross-pollination could occur. The company had also offered to undertake any monitoring activities BUWAL considered necessary. AgrEvo has until early May to decide whether to appeal.

Georg Karlaganis, head of BUWAL's biotechnology division, says the office is keen "to set a high standard". He says that the grounds for rejecting the AgrEvo patent are anticipated changes in a new European Union (EU) directive on the deliberate release of GM organisms. Though Switzerland is not a member of the European Union, its own rules governing the uses of genetic engineering are broadly similar to EU rules.

Proposed changes to the EU directive include a seven-year monitoring period for commercially grown crops. But regulations for field trials will not change substantially under the proposal, and licences, with or without monitoring requirements, will continue to be issued on a case-by-case basis, according to individual risk assessments.

Switzerland is revising its own rules, found in various federal laws, as part of the 'Gen-Lex motion'. This is intended to coordinate the laws relating to genetic engineering and to fill any gaps. It was initiated last year to take some of the heat out of debates leading up to the referendum on genetic engineering (see *Nature* 391, 312; 1998).

AgrEvo management fear that BUWAL will not issue any licences before parliament has approved the Gen-Lex motion, expected at the end of the year, for fear of setting a precedent. But Karlaganis says that BUWAL is not fundamentally against GM crops.

InterNutrition, a group representing the major Swiss food companies, says that it "regrets" the rejection of the two applications and that the move will harm the reputation of Swiss plant biotechnology research.

Isabel Meister, a biotechnology expert for Greenpeace International, sees the rejections as "a step in the right direction". Switzerland is one of the first countries to apply the 'precautionary principle', she says.

Alison Abbott

US processor rejects maize that EU won't take

[WASHINGTON] The third largest maize (corn) processor in the United States has refused to accept genetically modified (GM) maize that has not been approved for import by the European Union (EU).

In a letter earlier this month to grain elevators, A. E. Staley Manufacturing Co. of Decatur, Illinois, said that its plants would not accept GM maize hybrids that have not been approved by the EU. Such hybrids account for about 17 per cent of all GM maize being grown in the United States this year: about 7 per cent of the total US maize crop of some 80 million acres.

Farmers' and processors' trade associations say that the percentage of the crop affected is small and that Staley's move simply reflects a policy adopted by the industry a year ago.

Opponents of GM food see the Staley letter as evidence that public wariness of GM foods in Europe is beginning to force concessions, even as EU and US officials enter what are expected to be rancorous negotiations over labelling (see *Nature* 398, 641; 1999).

The Corn Refiners Association (CRA) says that customers have asked for EU-unapproved varieties not to be used at facilities where maize is processed for

How GM varieties are sweeping US cornfields

Year	Number of varieties of transgenic maize (corn) planted in the US	Percentage of US maize acreage
1996	one	0.75 per cent
1997	seven	9 per cent
1998	eleven	25 per cent
1999*	eleven**	39 per cent

*Numbers for 1999 are estimates
Source: Corn Refiners Association

**Seven are unapproved by EU

export. Consequently, it says, its members have asked grain merchants to help farmers avoid delivering EU-unapproved maize to refiners or export elevators.

Officials from Cargill Inc. and Archer Daniels Midland (ADM), the two largest US maize processors, said that they were already implementing the CRA policy. Martin Andreas, a senior vice president at ADM, says the company is asking farmers to channel unapproved crops directly to the domestic feed business. "With the cooperation of the seed companies and the farmers and the processors working together this problem can be handled," he says.

Phil Bereano, a professor of technology and public policy at the University of Washington in Seattle, argues that the processors are responding to "plain old simple capitalist supply and demand economics. What has been created is a very substantial market [for non-GM foods] with a sharp

border around it. Unfortunately, only European consumers will benefit."

A spokesman for the National Corn Growers Association says that the move should not be construed as a victory for anti-GM food activists. "If anything [the processors' position] is a victory for farmers," says Kevin Aandahl. "[It shows] we're responsive to customer needs while we're working through the cultural and trade issues. If anything it's an admission that we're all in this together."

The three affected varieties of GM maize are Monsanto's Roundup Ready, engineered for herbicide resistance; and two *Bt* varieties, engineered to produce a toxin made by a soil bacterium: Monsanto's *Bt* Xtra (formerly made by DeKalb); and AgrEvo's StarLink. The three, occurring alone or in combination in seven seed products, are expected to be planted on about 5.2 million acres in 1999.

Meredith Wadman

Swedish minister rebuilds scientists' trust

[STOCKHOLM] Sweden's new science minister, Thomas Östros, is moving to strengthen links with the science community that had been severely strained during the tenure of his predecessor, Carl Tham.

At a press conference in Stockholm last week, Östros confirmed that he would not be making any further changes to the management of the Swedish Foundation for Strategic Research, which Tham had wanted to be run entirely by politicians.

Östros also announced that government funding for research would increase by five per cent next year. And he confirmed that his government was keen to support curiosity-driven research based on an agenda set by the scientific community.

The decision on the research foundation ends more than 20 years of conflict. Östros' first peace offering was the presentation of a gold medal, *Illis Quorum* — for outstanding achievements in the arts and sciences — to Ingvar Lindgren, the outgoing executive managing director of the foundation, at a recent seminar to celebrate the foundation's fifth anniversary.

Together with two other 'strategic foundations', it had been created in the early 1990s by the then conservative government, using money from a fund that had been set up to give employees a share of companies' wealth.

At his first press conference in 1994, Tham had said that his top priority was to change the statutes of these foundations and to place all their funds under direct political control. But Östros said at the anniversary seminar that he was happy to leave the current arrangements as they were.

The dispute has its origins in the 1970s. The Swedish Labour Organisation suggested that a Workers' Fund — controlled by the trade unions — should be set up out of a tax on the profits of private companies, and the money used to buy shares in the companies for the benefit of employees.

The fund, strongly supported by left-wing political parties, was finally set up in a modified form in 1984. But when the conservatives came to power in 1990, they took the money and used it to create a number of independent, private research foundations (see *Nature* 360, 512; 1992).

The foundations were run by independent experts, and board members appointed their own successors. The conservatives moved more than SKr10 billion (US\$1.2 billion) out of the public domain into the private sphere this way.

After the social democrats regained power in 1994, Tham tried to change the status of the foundations and make their money public again. He failed, but succeeded in changing the composition of their boards and the way they were recruited. They now

consist of a mixture of scientific experts and politicians, all appointed by the government.

A public investigation set up by Tham proposed that the boards should consist solely of members of parliament. This was widely opposed by the research community.

Östros's statements suggest that there will be no more changes in the way the foundations are run. At his press conference last week, he repeated his wish to strengthen the dialogue with the scientific community. He confirmed that he regards the controversy around the strategic foundations as settled.

"We now regard the foundations as a belonging to the family of public agencies for funding science," he said, adding that he thinks the foundations are a valuable new way of financing science.

Only SKr15 billion of the country's SKr58 billion science funding comes directly from the government — the rest is from industry, the military and the strategic foundations. A five per cent increase in government funding will give science an extra SKr779 million by the year 2002. The money will be used to strengthen basic research and education within the technical and natural sciences.

Though this looks like a minor increase, it is a remarkable shift of focus from applied to basic research. It has been welcomed by scientists who were critical of a public-funding policy that favoured applied research.

In presenting the bill, the minister said he was confident that curiosity-driven basic research was the best guarantee of quality in science, and the most effective way of obtaining useful knowledge and commercially successful applications in the long term.

Östros said that the bill should be seen as a first step towards a fundamental reassessment of Swedish science policy which would produce a more long-term strategy, to be presented in a bill in 2000.

Swedish science councils, authorities, universities and organizations will be commissioned to work out research strategies of their own, and to look at the needs of developing Swedish science. The research councils will also be instructed to hold a series of conferences to gain background material for the new bill.

The public consultation that preceded Östros's bill suggested that all public funding agencies should be reorganized into four big research councils. But this suggestion met with strong protests from representatives of applied research.

Östros says he agrees on the need for a more effective way of distributing public money to science, but feels that the issue needs more investigation. No imminent change in the organization of the research councils is planned.

Östros has gained considerable backing from the scientific community during his seven months in office. One reason is his choice of members of the government's science advisory board. In addition to representatives from leading scientific institutions, the board includes officials from export- and research-dependent industries, as well as science students.

The appointment of immunologist Hans Wigzell from the Karolinska Institute as a personal science adviser to the government has also been widely welcomed. **Peter Sylwan**

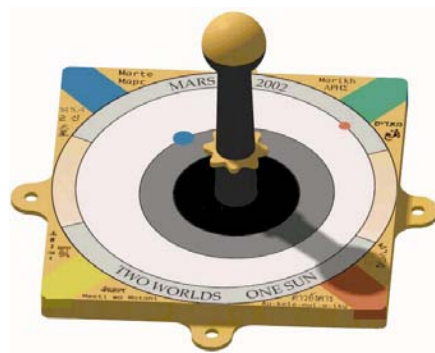
Mars sundial celebrates joy of discovery

[WASHINGTON] A Mars Sundial, about the size of a computer floppy disk, will be fixed to the Mars Surveyor 2001 lander due to touch down on the red planet in January 2002.

Viewers on Earth will be able to tell Martian time by tuning into pictures of the sundial taken by the Surveyor's panoramic camera and broadcast over the Internet.

Aside from its public relations value, the sundial will be used to calibrate the lander's colour camera. The idea came from Bill Nye, who as 'Bill Nye the Science Guy' hosts a popular children's science show on US public television.

Nye used children's design suggestions and enlisted the help of experts. These included Woodruff Sullivan, an astronomer and sundial expert at the University of Washington, and artist Jon Lomberg, who had a hand in the creation of the Voyager record sent into interstellar space with the Voyager 1 and 2 spacecraft in 1979.



Bright idea: pictures of the Mars sundial will be broadcast over the Internet from 2002.

Around the sundial's base is engraved a Voyager-like message that reads in part: "We sent this craft in peace to learn about Mars' past and about our future. To those who visit here, we wish a safe journey and the joy of discovery." **Tony Reichhardt**

Monitoring system planned for US biodiversity drive

[SAN DIEGO] US efforts to create a Biodiversity Observatory Network (BON) are gathering speed as part of a biocomplexity initiative launched earlier this year by Rita Colwell, director of the National Science Foundation (NSF).

The proposed network of up to 20 sites across the country would serve as 'weather stations' to monitor ecological and systematic factors over time, link data through computer systems, and compare findings to historic collections at museums and universities.

The BON proposal is part of a plan for a National Ecological Observatory Network (NEON), a broader monitoring system on which the NSF's governing body, the National Science Board, was briefed last month. Next week, the NSF will hold its latest workshop on BON in San Francisco, where scientists will try to resolve different approaches to biodiversity research.

Although funding amounts have not yet been proposed, federal officials say that BON could require budget requests of \$30 million to \$50 million a year for three years to create the network's infrastructure, plus five-year operational grants of more than \$500,000 a year for each site, and \$5 million to \$20 million annually for associated research grants.

Colwell has requested \$50 million for the biocomplexity initiative for the 2000 fiscal year, and intends to expand it further thereafter. But it is not clear that the Republican Congress, which killed a previous administration effort to found a National Biological Survey, will support such an expansion of support for ecology and systematic biology.

BON would be one of the most ambitious initiatives to date by the NSF's division of environmental biology, part of the directorate of biological sciences. The BON sites would be at locations owned by governments, universities or non-profit organizations.

Attempts to use the effort to survey private lands could stir political objections. Conservatives in the Congress have opposed funding for such studies, on the grounds that they encroach on private property rights.

"BON is a test component of NEON," says Bruce P. Hayden, professor of environmental science at the University of Virginia, who serves as the NSF's director of the division of environmental biology until July.

The NSF has sponsored two workshops on BON, with the third due to take place next week at the California Academy of Sciences. This is expected to address what one participant calls "the bond of marriage of ecology and systematics". Ecologists and systematists — who collect and classify specimens — "aren't ready to go to the altar yet", notes one



Diving for data: counting fish numbers — as here in Florida — will provide one source of input.

observer, adding that there is "a contentious" debate over their different approaches.

BON would record changes in the biota, reflect the chemical compositions of substances and assist in describing the origins of human diseases. The NSF hopes to combine the systematists' collecting capabilities with the ecologists' long-term analyses to produce an evolving picture of biodiversity at the sites. "If we can't work together, this thing won't float," says Hayden.

Many institutions and scientists are already involved in planning initiatives to lay the groundwork for BON, which NSF officials describe as moving at an "unprecedented" speed. Depending on funding, requests for proposals related to BON could come next year, say officials. For example, a consortium is forming to position its members to play key roles if funding is forthcoming.

Hatched in December at a conference on biodiversity modelling in San Diego, the nascent consortium involves universities in the United States and Mexico, museums in the United States and Canada, the San Diego Supercomputer Center, the NSF's 21-site Long Term Ecological Research Network, based at the University of New Mexico, and the National Center for Ecological Synthesis and Analysis in Santa Barbara.

Leonard 'Kris' Krishtalka, director of the University of Kansas Natural History Museum and a leader in forming the consortium, says the group is using an NSF grant that involves many of the same institutions as "a springboard" to be ready for BON. "No one institution has the mission, infrastructure and experience to do what is necessary," he says. "Solving the complexity of research riddles of biodiversity is going to require synthesis and collaboration."

Rex Dalton

NASA's plan to hire peer-review contractor raises scientists' fears

[WASHINGTON] A plan by the US space agency NASA to hire a single contractor to manage the peer review of all its research grant proposals has made some scientists worried that the quality of review will suffer during the transition period.

The agency hopes to invite small, minority-owned businesses this summer to bid for a 'consolidated peer review' contract to administer the outside evaluation of grant proposals in space science, Earth science, life and microgravity science, equal opportunity programmes, and education. Currently each office handles its own peer-review logistics, with about 5,000 proposals considered across the agency each year.

NASA intends to award a five-year contract worth more than \$50 million by next February. The agency's move to consolidate peer review follows a recent push to streamline its headquarters management and speed up the processing of grants (see *Nature* 393, 403; 1998).

But several scientists voiced their concern about the plan during last week's meeting of NASA's external advisory group for life and microgravity sciences. They worry that hard-won improvements to the existing peer-review system, which have raised the reputation of NASA experiments and brought them more in line with research conducted by the National Institutes of Health, will be jeopardized if the new contractor lacks experience.

"If we start going backward, what credibility has been built up may be lost," said Kenneth Baldwin of the University of California at Irvine, the chairman of NASA's life sciences advisory subcommittee. Gerard Faeth of the University of Michigan, who chairs the subcommittee for microgravity science, called the consolidation "probably the most critical issue for my constituency".

Those two disciplines have much at stake, as the office for life and microgravity sciences now gives responsibility for peer review — including selecting and presiding over review panels — to its contractor, Information Dynamics, Inc. of Virginia.

In contrast, NASA's space science office handles some of its peer-review duties in-house. Science research programme director Guenter Riegler says that consolidation could benefit his office by providing more contractor help to a small group of NASA civil servants. But managing the transition will be the difficult part, says David Bohlin, who runs the peer-review process for space science. "We have to keep the system operating while we [shift to a single contractor]," he says.

Tony Reichhardt

SSC spectre hovers over neutron project

[WASHINGTON] The \$1.4-billion project to build the world's most powerful neutron source at the Oak Ridge National Laboratory in Tennessee has fallen months behind schedule during its first year of construction, and faces major difficulties in obtaining funding for next year.

The Department of Energy is scrambling to sustain support from the Congress for the Spallation Neutron Source (SNS), the largest scientific facility that it has started to build since the abandonment in 1993 of the Superconducting Super Collider (SSC) in Texas.



Moncton: a new budget plan by July.

It has appointed a highly regarded new project manager, David Moncton of the Argonne National Laboratory, Illinois, who estimates that the project is "three to six months behind schedule" and has promised to prepare a new budget plan by July.

Last week Senator Bill Frist (Republican, Tennessee), an important backer of the project, condemned James Sensenbrenner (Republican, Wisconsin), chair of the House Science Committee, for his recent threat to suspend construction funds for the SNS (see *Nature* 398, 452; 1999). "The Spallation Neutron Source is a vital national project," said Frist. "Sensenbrenner's decision not to support funding for this project is short-sighted."

But Sensenbrenner, a 'budget cutter' noted for his outspoken attacks on projects favoured by the Clinton administration, is not alone in his concern about the project. Senior neutron scientists, a former official at Oak Ridge and an administration official who has supported the project in the past, all say privately that the project has been in trouble since at least last summer.

According to one of the officials, the project faces a credibility problem now because

the energy department did not come clean earlier about the problems Oak Ridge was facing last year. "They said they were hiring people, but there was no project there. I think they are more like a year behind."

The SNS was developed by the department with Oak Ridge as its preferred site after the Tennessee laboratory's more ambitious plan for a reactor-based facility — the \$3 billion Advanced Neutron Source — was abandoned in 1995 (see *Nature* 373, 460; 1995).

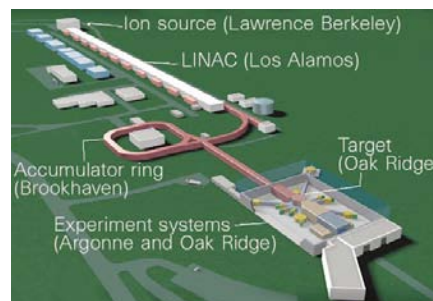
The new plan called for the SNS to be built by five laboratories in collaboration: Los Alamos in New Mexico, Brookhaven in New York state and Lawrence Berkeley in California, as well as Argonne and Oak Ridge. This was intended to compensate for Oak Ridge's lack of knowledge of all of the technologies needed, and to boost political support for the project outside Tennessee.

But, according to an independent review commissioned by the energy department in January, the project office at Oak Ridge had been unable to appoint a qualified technical director or to "assert technical leadership" of the whole project. Existing staff had "insufficient experience in the management and leadership of large, complex technical projects," the review said.

The department reacted quickly, appointing Moncton in place of Bill Appleton as project manager in March. "This is the new, fast response Department of Energy!" says Ernie Moniz, the energy under-secretary. "We had a new project manager in two months, and he's already doing an outstanding job."

Moncton built the Advanced Photon Source at Argonne — a project almost as large as SNS — and worked with neutrons early in his research career. He believes the facility can be built on time and within budget. "The most important thing is to attract people to Oak Ridge with expertise in construction and in accelerators and to integrate them with the partner labs, as if everyone was working for the same employer," he says.

Sensenbrenner has praised Moncton's



Coming together: layout of the SNS, combining expertise from five collaborating laboratories.

appointment. But he remains worried about a \$35-million 'use tax' that the state of Tennessee plans to levy on the project, as well as management and staffing problems. "I'm concerned that this is starting to establish a pattern of slippage," he says. "What this programme needs is some tough love. I'm darned if there's going to be another SSC fiasco on my watch."

Opponents of Sensenbrenner accuse him of attacking the project because it is in the home state of Al Gore, the likely Democrat nominee in the next presidential elections. Sensenbrenner, they point out, needs to curry favour with the House Republican leadership, which he hopes will promote him to chair the Judiciary Committee in the next Congress, and the leadership is running a 'get Gore' campaign to attack the vice-president on every front.

But the project's problems are real enough. It has been difficult to hire senior staff because the top management at Oak Ridge, including the director, Alvin Trivelpiece, will probably leave next March when Lockheed Martin, the contractor which manages the laboratory for the energy department, is expected to be replaced.

Appleton, the former SNS project manager who is now deputy director of Oak Ridge, denies that the SNS was as much as three months late under his charge. "The project was judged in January to be OK," says Appleton, a respected neutron scientist and administrator who says that "too much has been made" of problems on the project.

Trivelpiece says it was hard to keep the project on schedule because it is illegal to prepare for the use of funds until they are actually appropriated. "The money we had was parsed out in the most intelligent way possible," says Trivelpiece, who stresses that the energy department was kept fully informed of the project's status.

Asked who was responsible for the project running late, Martha Krebs, assistant secretary of energy, says: "What we've done is to make sure we don't get any further behind. I don't think it is useful to point fingers."

Colin Macilwain

The many applications of spallation neutrons

[WASHINGTON] The Spallation Neutron Source being built at Oak Ridge (see above) would have a power rating of 2 megawatts — more than 10 times greater than that of ISIS in the United Kingdom, currently the world's best neutron spallation source.

A spallation source generates pulses of neutrons by firing a proton beam at a heavy-metal target — made of

liquid mercury at the planned US facility — which then gives off neutrons at a wide range of energies.

Materials scientists, condensed-matter physicists and structural biologists use neutrons to study crystalline and molecular structures. Although more difficult to generate than X-rays, they are preferred for some applications because they

pick up images of lighter atoms which X-rays miss.

The average flux of neutrons produced by a spallation source is weaker than that delivered by other neutron sources based on nuclear reactors. But skilled experimentalists can make use of the sharp pulses of neutrons at the variety of energies that a spallation source will deliver.

C.M.

Japan moves to evaluate its engineers

[TOKYO] Japan is to set up a body to evaluate engineering and technology departments at universities in an effort to raise teaching standards to Western levels.

The move is a response to industry's concern about the poor quality of Japanese engineers (see *Nature* 393, 724; 1998). Because Japanese universities lack an adequate system of evaluation, companies say that they have been unable to determine the standard of engineering graduates.

The new body, Japan Accreditation Board for Engineering Education (JABEE), will be launched tomorrow (30 April) by the Japanese Society for Engineering Education (JSEE), which represents both industry and the academic community.

Modelled on accreditation bodies in the West, such as the US Accreditation Board for Engineering and Technology, JABEE will certify engineering, technology and applied science programmes according to the quality of teaching and curricula that are provided by the universities.

In addition to basic training in engineering and technology, JABEE will require such programmes to include teaching in humanities, such as ethics and communication studies, as well as English-language training. The accreditation process will officially start in

2001 after one to two years of experimental operation, with mutual accreditation by US and British accreditation bodies.

Some are concerned that such a system may not be accepted by universities, which have in the past been generally reluctant to endorse external evaluation (see *Nature* 397, 378; 1999). But Koji Harada, an executive director of JSEE, says: "This is no time to argue whether to introduce the evaluation system or not. Engineering is a global business, and Japan is the only developed country which does not have an internationally recognized standard for engineering education."

Despite a recent proposal by the Asia Pacific Economic Cooperation (APEC) forum to introduce a professional recognition system for engineers, called APEC Engineers Register, Australia and New Zealand are the only member countries which have internationally recognized accreditation bodies for engineering education.

Under the current proposal, 'APEC engineers' would have to graduate from universities approved by recognized accreditation bodies, and have at least seven years' professional experience in engineering.

"The problem with most Japanese universities is that technical training is not emphasized enough in their engineering pro-



grammes," says Harada. "Since engineering is regarded as an academic subject, most engineering graduates are not equipped with technical skills required by industry. Strictly speaking, engineers in Japan cannot be regarded as such in international terms."

Many blame this situation on the collapse of Japan's lifetime-employment system, which invests heavily in training new graduates. With the recent economic crisis, increasing numbers of companies are cutting back on training to save money, and are abandoning lifetime employment for contract-based employment.

Asako Saegusa

University libraries put pen to paper in journal pricing protest

[MUNICH] Leading specialist science-journal publishers have responded cautiously to an open letter signed by German, Austrian, Swiss and Dutch university libraries asking how they propose to keep prices affordable in future.

The letter was sent out in January, and pointed out that the announced increase in the price of journal subscriptions in 1999 was significantly above that of library budgets. The highest increases were from MCB University Press (27.3 per cent), Wiley (20 per cent) and Elsevier Science (19 per cent).

Most of the publishers replied by saying that their pricing policies for 2000 and beyond are not yet fixed. Some, such as Wiley, indicated that they were prepared to discuss the issue with libraries in the next months — a move welcomed by librarians, who say that publishers usually refuse to discuss pricing policy with them.

Elsevier promised to keep price rises next year below 10 per cent, but this statement has not reassured librarians, who still find the increase too high. Göttingen University librarian Elmar Mittler, for example, points out that although library budgets, which are paid out of public funding, are not necessarily falling, any increase is usually only a few per cent. "Last year we fought for, and won, a sup-

Price increases and impact factors for some specialist science journals

Title	Publisher	Sub. Price (DM)		Impact factor
		1998	1999	
Cell	Cambridge Cell Press	1,220	1,329	37.3
Neuron	Cambridge Cell Press	1,120	1,120	15.8
EMBO Journal	IRL/Oxford Univ. Press	2,144	2,325	12.6
PNAS	Academy of Sciences	1,437	1,904	9.0
Angewandte	Chemie Wiley/VCH	2,398	2,898	8.6
Journal of Biological Chemistry	Soc. of Biochemistry and Mol. Biol.	3,200	3,200	7.0
Biochemistry	Am Chem. Soc.	4,358	5,100	4.6
Glia	Wiley/Liss	3,600	3,870	3.8
FEBS Letters	Elsevier	6,065	6,879	3.5
Brain Research	Elsevier	24,404	27,228	2.1
Gene	Elsevier	12,137	12,817	1.8

Table taken from *Laborjournal*, Freiburg

plementary fund of DM1.2 million (US\$651,200) to keep up subscriptions at Göttingen University library. But even so, we still had to cancel 150 subscriptions," he says.

Derk Haank, chairman of Elsevier Science, told *Nature* that Elsevier has been forced to introduce high price rises because of changes in exchange rates. Also, he says, the rising number of submissions to some journals means that they have to increase in size.

The librarians' letter points out that market forces do not operate in the world of research publications — researchers need access to many journals, and most are con-

trolled by a small number of large publishing houses. Electronic access to these journals does not as yet offer a solution to high prices, as publishing houses tend to offer all-inclusive deals for paper and electronic forms.

Reinhardt says scientists need to change their attitudes towards publishing and boycott over-expensive journals. But an article in this month's *Laborjournal*, a German magazine for biological scientists, says researchers select their journals more on impact factor than price. The article points out, separately, that impact factor often correlates inversely with price (see table).

Alison Abbott

Scientists split on US smallpox decision

[WASHINGTON] Reversing a position it took as a member of the World Health Organization (WHO) in 1996, the Clinton administration announced last week that it would preserve one of the two known stores of live smallpox virus, housed at the Centers for Disease Control (CDC) in Atlanta.

A spokesman for President Bill Clinton said the decision has been taken on the grounds that live virus is essential for the development of antiviral medicines and novel vaccines to protect the population in the event of accidental or terrorist release of smallpox.

According to a White House statement, "[It] reflects our concern that we cannot be entirely certain that after we destroy the declared stocks in Atlanta and Kotskovo [in Russia] we will eliminate all the smallpox virus in existence. We have a responsibility to develop the drug and vaccine tools to deal with any future contingency."

But the announcement has rekindled a long-standing dispute among scientists and public-health officials as to how best to deal with the stores of a virus that causes a disease declared by WHO to be eradicated in 1980, after a 23-year campaign.

The administration said that a report published in March by the Institute of Medicine on the potential research uses of the live virus had influenced its decision. That report concluded that the most "compelling" need for long-term retention "would be for the development of antiviral agents or novel vaccines to protect against a re-emergence of smallpox due to accidental or intentional release". It did not pronounce on the advisability of destroying or keeping the stocks.

The United States and 189 other countries of the World Health Assembly, WHO's governing body, agreed in 1996 that the



Keep the pox at bay: an unimmunized population would be vulnerable if smallpox re-emerged.

remaining stocks of the virus — housed in high-security facilities at CDC and at the State Centre of Virology and Biotechnology in Kotskovo — should be destroyed this June. But they also agreed that WHO should reaffirm the decision when it meets next month.

In a poll carried out by WHO last year, 74 of 79 countries responding supported destruction. Russia opposed it, saying that valuable research remained to be done on the virus. Britain, France, Italy and the United States were undecided.

Last week's US decision drew criticism from proponents of destruction. They argue that the risks of maintaining the stock outweigh the potential scientific benefits. "Anything that can be done to mitigate against the possibility of that virus being released in the population again should be done," says Donald A. Henderson, director of the Johns Hopkins Center for Civilian Biodefense Studies.

Henderson was formerly a senior science adviser to the Clinton Department of Health and Human Services, and chaired a joint committee of his department and the Department

of Defense that examined the issue in 1995. He charges that a desire to maintain the stock in order to be able to retaliate in the event of a biological attack is behind the administration's reversal, which he claims emanated from the defence department.

Charles Carpenter, a professor of medicine at Brown University in Providence, Rhode Island, who chaired the Institute of Medicine committee, also favours destruction. "I don't feel that we have any reason to try to block its destruction if 99 per cent of the other nations would like to have it destroyed," he told *The Washington Post*.

But Joshua Lederberg, a professor emeritus at Rockefeller University and a long-time adviser to the US government on biological warfare and infectious-disease issues, applauds the decision. "We know absolutely nothing about the potential for the re-emergence of smallpox or its next cousin," he says.

In the event of an accidental or intentional reintroduction, says Lederberg, an unimmunized population would be extremely vulnerable. "The only thing we have left is to treat it once it has gotten hold" with antiviral drugs that are as yet undeveloped.

Lederberg calls the agreement for stock destruction an unenforceable, "feel-good" measure. It is backed, he says, by "people who aren't in close touch with the microbiology".

Lederberg says countries should preserve stocks under an agreement requiring declaration of possession, inspection of facilities, and research to develop antiviral drugs, and should make provisions for biosafety under the aegis of an international advisory group.

Scientists have also argued that it is impossible to anticipate the questions that might be posed about the virus in 10 or 20 years in areas like human immunology. Similarly, it is not known what tools — perhaps a suitable animal model — might then be available to address them. Without intact virus, they argue, the door to those questions would be shut forever.

In contrast, opponents call the search for antiviral medications impractical and hugely costly, with little chance of success and no adequate animal model available. There are other practical constraints, too, in working with some 450 frozen virus samples housed in a level 4 biosafety laboratory at CDC.

"There are great difficulties in carrying out research with smallpox, especially in terms of the competing priorities we have for the use of our space," says Brian Mahy, director of the Division of Viral and Rickettsial Diseases at CDC. He points out that the centres also need to accommodate research on other dangerous viruses, from Ebola to haemorrhagic fevers. "I think only a limited amount of work on smallpox is likely to be done in future."

Meredith Wadman

Radioactive leak at Indian power station

[NEW DELHI] The Indian Atomic Energy Commission (AEC) has admitted that eight workers were exposed to "mild radioactivity" last month. The incident happened when about six tonnes of heavy water leaked during the inspection of a coolant channel tube at the Madras Atomic Power Station.

The 220-MW reactor was under routine maintenance when the leak occurred. The incident — rated 1 on the international nuclear events scale of 0 to 7 — is the first since a fire destroyed the control room of a power reactor at Narora, 150 km from Delhi, six years ago; that rated 3 on the scale.

Neither the AEC nor the Atomic Energy Regulatory Board (AERB) reported the incident. It only came to light only after a newspaper broke the story two weeks later,

quoting the workers who had been treated in hospital.

AEC chairman Rajagopalan Chidambaram dismissed the event at a press conference in Mumbai (formerly Bombay) last week as of "no safety significance". He claimed that the workers, who were exposed to radioactive tritium, only received three times the amount of radiation they would have normally received in a week.

AERB says the leak was caused by failure of the sealing plug in the equipment used to examine the coolant channel by remote control. It says that a committee has been set up "to investigate the root cause, to review the design of the special seal plug of the inspection equipment, and to suggest remedial measures to avoid recurrence of such incidents."

K. S. Jayaraman

US veterans' affairs department sets up research watchdog

[SAN DIEGO] The US Department of Veterans' Affairs (VA) is to set up an independent Office of Research Compliance and Assurance to review all its studies nationwide, following the revelation of major problems in the management of research in Los Angeles.

The formation of the office was announced last week as a congressional hearing was held into disclosures about the conditions under which cardiology tests were made on veterans without appropriate consent at a large VA hospital in West Los Angeles (see *Nature* 398, 448; 1999).

Nearly 1,100 research projects at the VA health and research system in Los Angeles were shut down last month after the consent problems were publicized. Since then, officials say, some basic research projects and animal studies have restarted.

At the hearing, VA officials disclosed that they are also investigating allegations of informed consent problems at centres in Philadelphia, Pennsylvania, Cincinnati, Ohio, and Tampa, Florida. The new research office will examine scientific practices in

studies costing \$1.1 billion a year at 170 VA institutions around the country.

Scottish parliament 'will name science champion'

[LONDON] The Scottish Labour Party has promised that if it wins the forthcoming elections for the first 'devolved' Scottish parliament it will appoint an eminent scientist as the country's 'science and technology champion'. The holder of the post will be responsible for promoting Scottish science, and for bringing together academics and business people.

The proposal forms part of the party's science and technology strategy announced last week. Wendy Alexander, Labour's finance and industry spokesperson, said that Scotland had a long and proud scientific tradition, and had produced many famous scientific names. "My aim for the future is that we also use our science to produce famous products and more jobs," she said.

Lack of funds grounds Russia's satellites

[MOSCOW] Budgetary restrictions mean that "not a single Russian scientific satellite is likely to be launched this year", according to Nikolai Anfimov, director of the Central

Scientific Research Institute for Machine Building, the main scientific body of the Russian space industry. Russia only has four satellites left in orbit capable of taking astrophysical measurements.

Anfimov says that new types of scientific satellites are being designed, but the lack of finance is preventing them from being built and launched. He points out that, although in the past numerous Russian satellites have carried foreign scientific equipment, two US satellites launched last year towards Mars use devices that were made in Russia.

Japan's postdocs could win greater freedom

[TOKYO] Japanese postdocs would be helped to establish themselves as independent researchers under a plan proposed last week by a working group set up by the government under the Science and Technology Agency. Postdoctoral researchers would be given greater control over research funds, and the number of research posts at universities and national laboratories would be increased.

The working group is calling for the proposal to be included in the second phase of the government's five-year science and technology plan, due to start in 2001. The 'super postdoc programme' has been drafted

in response to growing concern at the lack of research positions for postdocs. According to the latest statistics, the number of postdocs in Japan is expected to total 10,187 by next March.

India's missile 'ready to carry nuclear warhead'

[NEW DELHI] India's intermediate range ballistic missile, Agni-2, is ready for deployment with a nuclear warhead, says A. P. J. Abdul Kalam, chief of the defence research development organization which developed it. Agni-2 made a test flight on 11 April "in an operational configuration".

In an interview on state-owned television, Kalam said that scientists have fulfilled "the task given to them", and that it was up to the government to deploy the missile. Intended to counter China's nuclear might, Agni-2 does not require any more flight tests, according to Kalam.

Korea's president pledges action as scientists strike

[TOKYO] South Korean president Kim Tae-Chung promised last week to promote plans to find work for unemployed scientists, to provide "reasonable" payment for scientists and technicians, and to increase the

proportion of government spending devoted to research and development to 5 per cent by the year 2002.

Kim was addressing a meeting at the Korea Advanced Institute of Science and Technology held to mark the thirty-second National Science Day. His remarks coincided with a nationwide strike by more than 3,000 scientists and technicians in protest at government plans to introduce large-scale redundancies in state-run research centres.

Yi Kuang, chairman of the Korean Scientists and Technicians Union, said in a speech that the government's moves to restructure its research organizations meant that "the research environment has greatly deteriorated and morale among scientists has dropped sharply".

Planning delay 'hinders UK medical research'

[LONDON] The British government could do more to enhance biomedical research by ensuring the public acceptability of animal research, and that planning decisions are taken in the national interest, according to Michael Dexter, the director of the Wellcome Trust.

Referring to Wellcome's battle to build an extension to the Genome Campus at Hinxton, outside Cambridge, Dexter

complained that "18 months since our planning application was submitted, we still have no assurance that we will be able to extend our campus" (see *Nature* 398, 355; 1999). Speaking at a meeting of the Parliamentary and Scientific Committee this week, Dexter added that reassurance on the use of animals was needed so that properly regulated research could be "carried out without a climate of fear and intimidation".

Nuclear agency passes buck on anticancer drug

[WASHINGTON] The US Nuclear Regulatory Commission (NRC) is attempting to avoid paying for stockpiles of the drug potassium iodide, which decreases the risk of thyroid cancer from nuclear fallout, in states with nuclear power plants. The stockpiles are estimated to cost \$3 million a year.

The commission agreed to pay for such stockpiles in 1997. But last week its commissioners approved a plan to shift responsibility for payment to the Federal Emergency Management Agency, arguing that its own budget "offers little margin to divert resources to new initiatives".

The NRC will receive public comments on the proposed change for 90 days after a forthcoming announcement in the *Federal Register*.



The full text of items on this page — and further details and background information about the Unesco/ICSU World Conference on Science, to be held in Budapest from 26 June to 1 July — can be found on *Nature's* website at <http://www.nature.com>

Ethics group to look at energy, water and the information society

[LONDON] Unesco's World Commission on the Ethics of Scientific Knowledge and Technology (COMEST) is due to prepare its contribution to the World Conference on Science at its first meeting, being held in Oslo this week at the invitation of the Norwegian government.

During the three-day meeting, COMEST will undertake what Unesco describes as "a first exchange of views on ethics and the information society". Public round-table discussions are also planned, which will bring together representatives of international and non-governmental organizations, as well as players from the public and private sectors. The round-table themes will be ethics and energy, ethics and free water resources, and the ethics of the information society.

Such areas, says Unesco, reflect COMEST's mandate to serve "as an intellectual forum for the exchange of ideas and experience; to detect the early signs of risk situations; to fulfil an advisory role for decision-makers, and to promote dialogue between scientific communities, decision-makers and the public".

COMEST was set up in May 1988 by Unesco, with 18 members appointed by the director-general, to advise the organization and to serve as an intellectual forum for the exchange of ideas and experiences and to promote dialogue between scientific communities, decision-makers and the public. COMEST chair and former president of Iceland, Vigdís Finnbogadóttir, is due to address the opening session of the meeting.

Report: <http://helix.nature.com/wcs/a27.html>

How biotechnology could be Africa's route to riches

[HAMMAMET, TUNISIA] Africa-wide investment in biotechnology could be the continent's route to prosperity, says Thomas Odhiambo, founder and outgoing president of the African Academy of Sciences.

Addressing the academy's fifth general conference in Hammamet, Tunisia, this week, after 13 years at the head of the organization, Odhiambo said that African scientists and entrepreneurs need to find "niche areas" where their investments have a stronger chance of succeeding — such as the commercialization of genetic resources.

Odhiambo said that, while many countries struggle to find products and markets for their nascent biotechnology industries, Africa has a genuine competitive advantage, given that it owns the world's largest stock of plants with known medicinal properties.

He added that scientists and policy-makers in Africa need to modify their approach to biotechnology, from seeing it as primarily a source for the continent's future food needs to building a major knowledge industry. Africa's transformation from a recipient of development aid to a major competitor in world markets relies on such a change, said Odhiambo.

The outgoing president's comments are expected to form the basis of one of the key recommendations from the Africa region for the World Conference on Science in Budapest. The Tunisian conference's recommendations for the Budapest meeting, known as the 'Tunis Declaration', are expected to be finalized next week.

Odhiambo's suggestions indicate a more

proactive response from leading African scientists to bioprospecting by overseas multinationals. Until now, the region's leading scientists had called for changes to global intellectual property legislation as the main route to protecting indigenous knowledge.

His comments were broadly echoed by Ali Mazrui, professor emeritus of African studies at Cornell University, New York. But Mazrui warned the academy's fellows not to repeat past mistakes when attempting to embrace new technologies.

The poor state of research and higher education in Africa, Mazrui contended, had come about partly because the architects of these institutions had modelled them mostly on what they saw in Western Europe, taking little account of different linguistic and cultural environments. "An African renaissance somehow needs a 'reindigenizing' component," Mazrui told the conference.

The academy's new president is Mohammad Hassan, a mathematician and executive director of the Third World Academy of Sciences. Hassan says that one of his first tasks as president will be to persuade African governments to make more use of their senior scientists, for example through advisory committees in the areas of water management, agriculture and drought — the continent's three most pressing problems.

Another priority, says Hassan, will be to encourage centres of research excellence in Africa to help build up similar centres in neighbouring countries by passing on advice and engaging in collaborative projects.

Full report: <http://helix.nature.com/wcs/a26.html>

Virtual labs offer chance for South to beat the science brain drain

[LONDON] Virtual laboratories, based on the extensive use of electronic communications, could be a valuable device for combating the brain drain from the South, according to three researchers at Abdus Salam International Centre for Theoretical Physics in Trieste, Italy.

But Enrique Canessa, Fulvio Postogna and Sandro Radicella add that developing countries have a long way to go before they can fully exploit the Internet's potential.

An analysis of articles published on the Los Alamos e-preprint archives in condensed-matter physics and high-energy physics (theory), revealed 88 and 50 e-preprints respectively from groups of

authors working in different countries. In each archive, however, only one e-preprint has resulted from a collaboration between developing country institutions alone.

"It seems unlikely that virtual laboratories will be widely adopted by developing country scientists in the near future," say the authors. One reason is a lack of adequate computing literacy in the academic communities of countries new to the Internet. Other important factors are human diversity and age differences.

"Participation in interactive sessions from the South should make it easier to do science, not become another barrier to surpass," say the authors. They hope to see

the administration of 'virtual laboratories' simplified "so they can be run by scientists for scientists in their own countries".

But they note that the limited bandwidth of the few available telecommunication lines in developing countries causes congestion and often makes access too slow to use.

They recommend that Internet access grants or subsidies should be made available to scientists from less-privileged countries. "The 'virtual lab' approach could become a worthwhile service that helps to reduce scientific isolation while filling the need to transfer knowledge to the South in an unprecedented way," they conclude.

Full text: <http://helix.nature.com/wcs/c12.html>

Why Japan's professors fear evaluation

Sir— You pointed out the necessity of external review of Japanese universities (*Nature* 397, 371; 1999). It is true that external evaluation works efficiently if universities have equal opportunities in research — that is, if the 'losers' after evaluation have a chance to become 'winners' one day. But, because of the hierarchy among Japanese universities, the losers will have no such chance. This explains why professors in reputable universities are scared of external evaluation.

Table 1 shows a comparison of the two leading national universities, the University of Tokyo and Kyoto University, with two typical local national universities, Niigata University and Tottori University; the best two private universities, Waseda University and Keio University; and the best private science university, the Science University of Tokyo. Waseda and Keio have the best reputation in literature, economics and politics. There are remarkable differences between the scientific grants obtained by the best national universities and the rest.

But lower grants do not necessarily imply lower scientific quality of faculty members. They are more likely to reflect a lack of time for research, because of heavy teaching duties, especially in private universities. And note the differences in the ratios of students to faculty members. As a result of the heavy

Table 1 **Comparison of Japanese national and private universities**

University	Grants per faculty member*	Student/faculty ratio	Government subsidy per student*	First-year tuition fee*
Univ. of Tokyo	28,000	4.0	19,000†	6,200†
Kyoto Univ.	26,000	5.0		
Niigata Univ.	5,500	9.2		
Tottori Univ.	4,000	7.7		
Waseda Univ.	4,100	29.4	1,600	13,000‡
Keio Univ.	7,500	17.9	2,700	13,000‡
Science Univ. of Tokyo	3,400	18.5	1,800	11,000‡

*US\$; †Average for national universities; ‡Science and technology faculty.
Source: *University Ranking '99*, published in the *Asahi* newspaper.

teaching load, good scientists at private or local national universities rarely publish important papers.

The income of private universities is almost completely dependent on tuition fees from students. Faculty members are not allowed to draw their salaries from scientific grants from the Ministry of Education, Science, Sports and Culture (Monbusho), and the private universities are not allowed to get overheads from the grants. National universities, on the other hand, are subsidized by the government without any assessment. Private universities receive only about one tenth of the subsidy per student that is given to national universities.

Given this unfair situation, who can expect fair competition in research? The situation is similar to the issue of the trade

imbalance between Japan and other countries. Although Japanese markets are officially declared open to foreign companies, they cannot gain 'fair' access. Similarly, it is impossible for private universities to compete with national universities in research.

This problem seems to be intrinsic to Japanese society. Recently, the Council on University Education, an advisory body to Monbusho, published a report proposing a plan to stimulate competition among universities. Amazingly, however, there is not a word in the report about problems caused by the hierarchy among universities.

Mitsuo Tagaya

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Some PhDs are more equal than others

Sir— One important aim of the European Union (EU) is to allow the free circulation of people among the different countries. In spite of this, countries such as Portugal demand that a PhD awarded abroad must be 'recognized' in Portugal before a researcher can have a university job.

The quickest way of gaining this 'recognition' is to 'register' the PhD, a process that involves the submission of the diploma, a copy of the thesis and a payment of up to 25,000 escudos, about US\$130, to a Portuguese university. Usually, PhD status is granted after 10 days. A more lengthy process known as 'equivalence' involves the re-evaluation of the thesis by a panel of Portuguese professors, as if the judgement of the original jury was not to be trusted.

Although everyone assumes these to be bureaucratic formalities, they do allow for the possibility of revoking a degree awarded by another EU university, and indeed the University of Lisbon has recently chosen to reject the PhD that I obtained at the

Technical University of Denmark in 1988. Are we really free to move between the universities of all EU countries?

Leonor Cruzeiro-Hansson

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Name and shame the scientific fraudsters

Sir— With regard to the recent coverage of fraud in science¹, I would like to suggest some remedial actions to counter fraud and malfeasance in British universities.

I am not alone in my anger and contempt for universities that abuse scientists and others^{2,3}. But it is difficult to obtain legal redress against a British university administration. A university with a royal charter has special status in common law that renders it invulnerable to prosecution in a county court.

An excellent survey of grievance procedures, by Don Staniford *et al.*⁴ of the National Postgraduate Committee, illustrates the problems clearly. The present system allows for complaints to be

investigated by a 'university visitor', appointed by the Privy Council. But trying to get the Privy Council to act on your behalf is like getting a live eel into a jar of Swarfega.

The cause of academic fraud and malfeasance is greed and conceit with relative impunity. My suggested remedies are, first, to end the chartered status of British universities. Second, to register all research students directly with their universities and not to their supervisors. Third, for science journals to fund a task force to maintain a database of all suspected and proven agents of fraud and malpractice, and to investigate and collect relevant information. The task force would act as an advice bureau for honest scientists, with the power of naming and shaming all proven cases in the journals. Fourth, for all research funding bodies to check with the task force before allocating grants.

James McComb

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1. Abbott, A. *et al.* *Nature* 398, 13–17 (1999).

2. McComb, J. *Nature* 387, 448 (1997).

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4. Staniford, D. <http://www.npc.org.uk/>

Ants, plants and antibiotics

Ted R. Schultz

The partnership between ants and their fungal gardens has a newly discovered third member – a bacterium, which grows on the ants' bodies and produces antibiotics to kill a parasite that infects their crops.

Roughly 50 million years ago in South America, a lone species of ant abandoned its primitive hunter-gatherer ways and, in a unique event in ant evolution, adopted an agrarian lifestyle. Entering into a partnership with a parasol mushroom, these agricultural pioneers learned to weed, manure and propagate their fungal crops, ensuring a reliable source of food. From this innovative ancestral stock arose the ant group Attini, of which there are now about 210 species, largely concentrated in wet, South American forests.

The Attini include the well-known leaf-cutting ants, in which the association (or 'symbiosis') between ants and fungi has become enormously successful. Colonies of some *Atta* species may contain eight million ants, with the collective biomass of an adult cow. These ants cut a cow's daily requirement of fresh vegetation, but they do not directly consume it. Instead, by chewing it into a pulp, they convert the vegetation into a substrate on which their fungal crops are grown. The fungus, in turn, produces specialized structures known as gongylidia, which serve as food for the ants. This arrangement has been called an "unholy alliance"¹, because it combines the ants' ability to circumvent plant antifungal defences (such as the waxy coatings of leaves, which the ants scrape away) with the ability of the fungus to subvert plant anti-insect defences (such as chemical insecticides, which are digested by the fungus, so are absent from the fungal tissue consumed by the ants).

The leaf-cutters have been known since earliest times — they are mentioned, for instance, in the Popul Vuh, the creation myth of the Central American Mayan civilization (300–900 AD). But the reason for cutting leaves was long misunderstood. In 1863, the British naturalist H. W. Bates incorrectly asserted² that "the leaves are used to thatch the domes which cover the entrances to [the ants'] subterranean dwellings, thereby protecting from the deluging rains the young broods in the nests beneath". The surprising truth was finally deduced by the naturalist and mining engineer Thomas Belt. In 1874 he wrote³: "I believe the real use [the ants] make of [the leaves] is as a manure, on which grows a minute species of fungus, on which

they feed; that they are, in reality, mushroom growers and eaters". Belt's revelation (which was independently discovered that same year by Fritz Müller⁴) marks the beginning of scientific studies into non-human agricultural symbiosis. But it now seems that biologists have vastly underestimated the true extent of this association. Instead of the commonly accepted two-part symbiosis between ant and fungus, Currie and colleagues⁵ report in last week's *Nature* that the attine symbiosis actually consists of three partners from three separate kingdoms — ant, fungus and antibiotic-producing bacterium — as well as a parasitic fungal 'weed' that infects attine gardens (Fig. 1).

Attine fungal crops are usually propagated from existing gardens. A foundress ant queen carries a small pellet of fungus from her mother's nest in her mouth. When she founds a new nest, she uses this pellet to start her new garden. Such vegetative (or clonal) propagation from one generation to the next

suggests that these ancient crops have strictly coevolved with their ant hosts. But phylogenetic and population-genetic studies of attine ants^{6,7} and fungi^{8,9} support an alternative picture, at least in the 'lower' Attini, which do not cut leaves. Here, ant colonies occasionally replace their clonal crops with free-living fungi acquired from outside the nest. Colonies also replace their resident fungi with crops obtained from other ants, so even distantly related ant species may sometimes share the same garden clone. The best long-term evolutionary strategy for most ant farmers seems to be cultivating a diversity of crops, rather than committing exclusively to a single one.

Such a strategy makes sense in the face of shifting environmental pressures, and Currie and colleagues⁵ have now identified a particularly important source of such pressure — a group of closely related, highly specialized parasites in the fungal genus *Escovopsis*^{10,11}, which infect the ants' fungal gardens. This is an ancient association — *Escovopsis* parasite species are found in gardens of just about all species of fungus-growing ants, but not, so far, in any other habitat. They are not transmitted from the parental to offspring nest; instead, they must infect every new generation of gardens from the outside, probably by hitchhiking on the ants' bodies. Once inside, the *Escovopsis* parasite bides its time at low frequency. But, like many other pathogens that are highly adapted to their hosts (including some human diseases), when the health of the garden is compromised, the parasite becomes virulent,

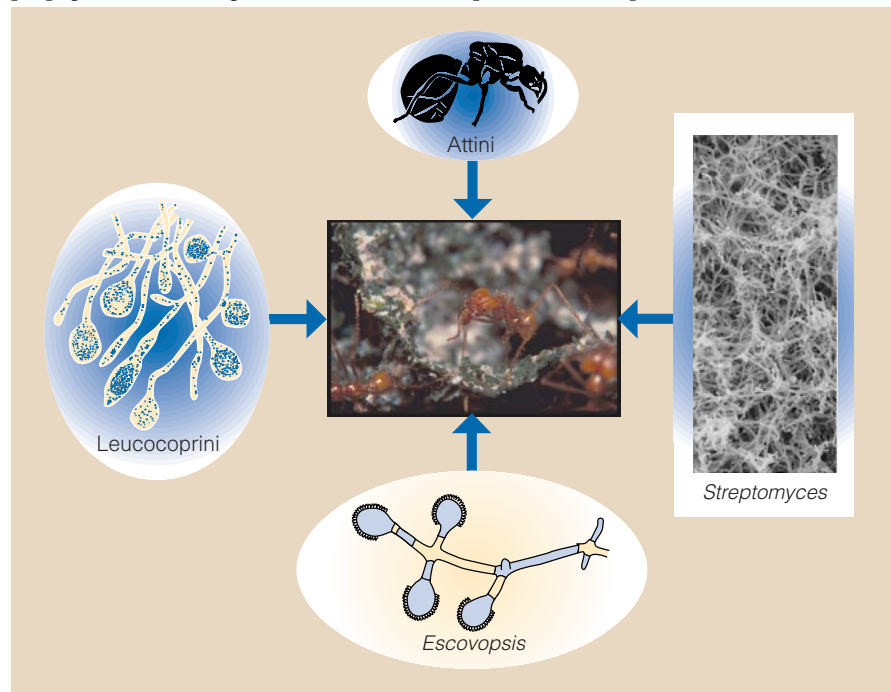


Figure 1 Ants use antibiotics to control garden pests. The attine ant cultivates a mushroom from the fungal tribe Leucocoprinini as a source of food. Currie *et al.*⁵ now show that there is another partner in this mutually beneficial relationship — a bacterium from the genus *Streptomyces*. This bacterium produces antibiotics to prevent infestation of the ant garden by the parasite *Escovopsis*, which is itself also a fungus.

DAN L. PERMAN

overgrowing and destroying the fungal crop in a short period of time.

In healthy gardens, Currie *et al.* show that *Escovopsis* is held in check by specific antibiotics produced by bacteria living on the bodies of the ants. It seems hardly a coincidence that these bacteria belong to the genus *Streptomyces*, from which over half of the antibiotics used by humans are derived. Like the parallels between ant and human agriculture⁹, understanding this use of antibiotics by ants could be directly relevant to human survival. For example, whereas humans have been using antibiotics for fewer than 60 years (longer if you consider the medicinal use of moulds in the ancient Far East, or among the Greeks and Romans), ants have been using them for 50 million years. Given that rapidly evolving pathogen resistance seems to be outpacing human antibiotic development, we might ask how the attine antibiotics have remained effective against the fungus-gardained pathogens for such a long time.

The answer probably involves an evolutionary 'arms race', in which garden pathogens and antibiotic bacteria (as well as fungal crops and ants) have evolved in tandem down various evolutionary pathways. The result is the particular assemblages of ants, fungi and bacteria that we encounter today. To understand how these assemblages came to be, we need to reconstruct and reconcile the evolutionary histories of each participant. Ideally, we also need to identify the closest free-living relative of the attine bacteria (probably an inhabitant of South American soils) and of the garden parasites (possibly a pathogen of free-living fungi).

Given the usefulness of the attine system as a model for symbiotic evolution^{1,8,9,12,13} and the parallels between ants and humans, a comparative biological study within an evolutionary-historical framework may reward us with theoretical insights into symbiotic evolution, as well as with practical insights for such diverse fields as medicine and agriculture. But none of these rewards will be realized unless we conserve the rapidly declining rainforests, which are home to these irreplaceable end-products of vast spans of evolutionary time. □

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two charge states of a 'single-Cooper-pair box' (see Box 1) by observing coherent oscillations between these states. Although the coherent quantum superposition of charge states was implied by several previous experiments⁵⁻⁹, this is the first time it has been observed directly. The audaciously simple idea behind the experiment is to align the energy levels of a Cooper pair (two electrons bound together in a superconductor) in the two electrodes of the Cooper-pair box using a voltage pulse of about 100 ps. When the levels are aligned, the Cooper pair starts to oscillate back and forth between the electrodes, and the oscillations are detected by monitoring one of the electrodes.

The question of whether the observed quantum coherence of the charge states is macroscopic or not is debatable in much the same way as the question of how many grains of sand are needed to form a sandpile. On the one hand, a Cooper-pair box has all the attributes of a macroscopic system. It consists of billions of atoms and the parameters of its charge states, most importantly their energies, are defined by the collective motion of the billions of electrons in the junction electrodes, and can easily be controlled by external signals. For instance, the charging energy depends on the geometric capacitance of the box, which reflects the collective screening properties of the electron gas in the box electrodes. Similarly, the amplitude of Cooper-pair tunnelling depends on contributions from each of the electrons in the box. This all points to an interpretation of the quantum coherence of charge states in the Cooper-pair box as macroscopic. On the other hand, the two charge states forming the coherent superposition differ in charge by only one Cooper pair. One Cooper pair tunnelling between these two states is identical to one electron tunnelling between two localized states, which is clearly a microscopic process. Because of this contradiction between the microscopic and macroscopic aspects of coherence of the charge states in the box system, it is probably appropriate to interpret this effect as 'mesoscopic' quantum coherence.

Besides its significance in the context of macroscopic quantum phenomena, Nakamura and co-workers' experiment⁴ also demonstrates a practical solid-state qubit for quantum computation. At present, there is an intensive search for realistic approaches to the problem of building a quantum computer. Despite impressive progress achieved in this direction by NMR spectroscopy of organic molecules in solutions^{10,11}, it seems to be impossible to scale up the few qubit gates provided by the NMR technique for a practical solution to the problem. In contrast, solid-state structures offer a much greater degree of control over design and fabrication, necessary for constructing larger-scale devices.

Several possible implementations of

Quantum computation

Solid-state qubits under control

D. V. Averin

One of the most significant developments in physics during the past 20 years is the clear and convincing proof that macroscopic systems, such as a superconductor with billions of electrons in it, can behave quantum mechanically. This conclusion changes the view, which was established in the early days of quantum mechanics, that there is a fundamental difference between the microscopic systems that obey quantum mechanics and the macroscopic world of classical physics. This has important implications for the conceptual foundations of quantum theory¹, in particular quantum measurement theory, which is frequently described in terms of the separation of the microscopic object and the macroscopic measuring device.

The most fundamental demonstration of quantum mechanics at the macroscopic level would be the coherent superposition of two distinct quantum states, which is a purely

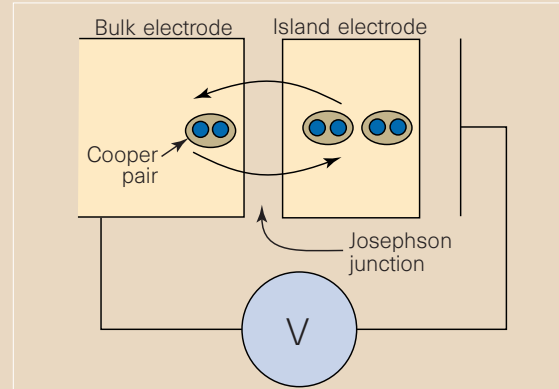
quantum effect and has no classical analogue. This effect also has implications for quantum computing, because a quantum two-state system represents a single quantum bit, or qubit, which is the elementary building block of a quantum computer. Superposition of information states in qubits should make quantum computers fundamentally more powerful than classical computers, a prospect that attracts the interest of computer scientists and physicists, as well as government agencies. As part of a quantum computer, macroscopic qubits would have the advantage of being more manageable than their microscopic counterparts.

For a long time macroscopic quantum coherence remained elusive, despite observations of other macroscopic quantum effects^{2,3}. On page 786 of this issue, Nakamura *et al.*⁴ report an important step towards its realization. The authors provide convincing evidence of quantum superposition of the

Box 1 A box for Cooper pairs

The Cooper-pair box shown in the figure consists of a small superconducting island electrode weakly coupled to a bulk superconductor. For the purposes of this discussion, each superconductor is simply a Bose condensate of Cooper pairs in which a single quantum state is occupied by a macroscopically large number of pairs. Weak coupling of the superconductors creates a Josephson junction between them. Another property of the Cooper-pair box is that the superconductors form an electric capacitor and the current in the Josephson junction changes the charge on this capacitor. The gate voltage V controls the potential difference between the electrodes.

Quantum mechanics tells us that, for any Bose condensate, the phase of the condensate wavefunction and the number of particles in the condensate are related by an uncertainty principle similar to the one relating the position and momentum of a quantum particle. If the phase behaves as a well-defined classical variable, quantum fluctuations in the



number of particles are large. And vice versa, if the fluctuations in the number of particles are squeezed, the phase uncertainty increases. This number-phase relation turns the Cooper-pair box into a simple macroscopic quantum system, with the Josephson phase difference and the charge of the box playing the roles of position and momentum. Transition between the regimes of well-defined charge in this system is driven by the charging energy of one Cooper pair as compared to the energy of Cooper-pair tunnelling. In a large box, the charging energy is small, and the current can transfer large number of Cooper pairs between the electrodes. In this case, the charge uncertainty is large and the phase is well

defined. When the box becomes smaller, the charging energy breaks the continuous current flow between the box electrodes into discrete transfer of individual Cooper pairs. For a very small box, the charging energy dominates and at most one Cooper pair can oscillate between the box electrodes, although the number of Cooper pairs in the electrodes is still macroscopically large. In this 'single-Cooper-pair box' regime used by Nakamura *et al.*⁴, the effectiveness of the Cooper-pair transfer depends on the energy difference between its states in the two electrodes of the box. The transfer becomes most effective when a gate voltage is applied to suppress the energy difference, leading to resonance and coherent oscillations. **D. V.A.**

solid-state qubits have been proposed in this context. The two major types of proposals are based on the dynamics of electrons in quantum dots¹² and on the dynamics of Josephson tunnel junctions^{13–15}, which are formed when two superconductors are separated by a thin insulator. Superconductivity is the result of the correlated motion of electrons in Cooper pairs, and tunnelling of these pairs across the insulating layer of the junction creates a supercurrent. The supercurrent gets its name from the ability to flow without resistance and dissipation of energy. This ability makes Josephson junctions attractive candidates for solid-state qubits.

The coherence of the Cooper-pair box

demonstrated by Nakamura *et al.*⁴ supports the idea of Josephson-junction qubits by realizing two basic elements of junction operation as a qubit. It is shown that the junction dynamics can be reduced to two states, and that the parameters of the two-state system — the energy difference between the states and their tunnel coupling — can be controlled externally. Control over the tunnel coupling is achieved by replacing one Josephson junction of the Cooper-pair box with a pair of junctions in a superconducting loop — a system that is sensitive to an external magnetic field. Magnetic flux through the loop changes the relative phase of Cooper-pair tunnelling in the two junc-

tions and modulates the total tunnel coupling. The ability to switch the tunnelling on and off, and to change the energy difference between the charge states by the gate voltage is sufficient to perform any transformation on the states of one qubit required for quantum computing.

The experiment also gives an idea of the strength of the decoherence in the Josephson-junction qubits. Decoherence occurs when a quantum system interacts with the environment, and is the main problem facing all quantum computers. In the experiment by Nakamura *et al.*, considerable effort is devoted to minimizing interaction with the environment — the Cooper-pair box is cooled down to about 30 mK, and is carefully shielded from external electromagnetic radiation. Nevertheless, coherence is observed for only about six cycles of the Cooper-pair oscillations. The shape of the oscillations (a constant oscillation amplitude that disappears abruptly after a certain time interval) indicates that the decoherence is caused by a low-frequency noise. The likely source of this noise is charged impurities in the substrate or tunnel barriers in the box structure. This 'background charge' noise varies strongly from experiment to experiment, and further studies should make it possible to suppress it significantly and to increase the coherence time of the Josephson-junction qubits. It will then become possible to study other, more subtle, decoherence mechanisms in these qubits. It seems natural to expect that the progress of experimental solid-state quantum computation will be difficult, but this first successful step gives hope that it will continue despite the most daunting problems. □

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Developmental biology

Legs to wings and back again

Lee Niswander

How do arms or wings (forelimbs) become different from legs (hindlimbs)? This biological problem has interesting evolutionary implications, and a handful of papers^{1–4} — including reports by Takeuchi *et al.*¹ and Rodriguez-Esteban *et al.*² on pages 810 and 814 of this issue — provide the first tantalizing molecular clues. They show that two transcription factors, Tbx5 and Tbx4, which are expressed in the developing forelimb and hindlimb, respectively, control the identity of arms and legs.

Takeuchi *et al.*¹ and Rodriguez-Esteban *et al.*² first show that if the Tbx5 protein is misexpressed in the chick embryonic hindlimb, the leg ends up looking more like a wing — it has three (as opposed to four) digits, an altered fibula, and feathers instead of scales (Fig. 1). These workers, as well as Logan and Tabin³, also show that when Tbx4 or Pitx1 (a bicoid-related homeodomain protein that is also restricted to the hindlimb) are misexpressed in the wing region, leg-like structures form (these have four digits, claws, an altered ulna, smooth skin or scales instead of feathers, and a leg-specific muscle pattern). Moreover, when Szeto *et al.*⁴ knocked out the *Pitx1* gene, expression of Tbx4 was reduced and the authors saw partial leg-to-arm transformations. These results indicate that Pitx1 positively regulates expression of Tbx4 in the leg.

But why are the limbs not completely transformed when these genes are misexpressed? Perhaps the specification of limb identity relies on a number of events, only a subset of which are affected by the loss or gain of a single transcription factor. Moreover, experimental limitations may reduce the scope of the transformations. The first limitation — timing of misexpression — is critical. Other studies indicate that the identity of fore- and hindlimbs is set early and retained, even if leg tissue is grafted to the wing (resulting in wings with toes and claws) or vice versa^{5,6}. Takeuchi *et al.*¹ used *in ovo* electroporation, which allows rapid expression of introduced DNA, yet they still found that the transformations were incomplete. The second limitation is continued production of the proteins that are normally expressed in the limbs. For example, when Pitx1 or Tbx4 are misexpressed in the wing, production of Tbx5 persists, and this may maintain wing identity. But when Tbx5 is misexpressed in the leg, expression of Tbx4 drops considerably. Again, transformation is incomplete, perhaps because expression of the upstream 'leg' regulator Pitx1 is unaffected.

It may be that the problem is with our expectations — loss of hindlimb identity may not necessarily be equivalent to gain of forelimb identity, and vice versa. In mice that lack Pitx1, expression of Tbx4 is reduced and the hindlimb comes to resemble a forelimb, despite the lack of misexpressed Tbx5. This result suggests that forelimb identity may be a 'ground' state, independent of Tbx5, that is overridden in the hindlimb by Pitx1 and Tbx4. It could explain why misexpression of Pitx1 or Tbx4 in the forelimb causes partial transformation to a hindlimb. So, perhaps the function of Tbx5 is not to regulate forelimb identity per se, but to repress hindlimb identity. In that case, it should be possible to develop forelimb structures in the absence of Tbx5. This theory can be tested by generating mice that lack either, or both, the *Tbx5* and *Tbx4* genes.

People with Holt–Oram syndrome, which is caused by reduced levels of Tbx5^{7,8}, do not show transformations in limb identity. But they do have malformed forelimbs, including loss or duplication of the thumb, and a reduced or missing radius. So it seems that Tbx5 is required specifically in the anterior skeletal elements of the forelimb. Increased levels of the Tbx proteins also appear to interfere with development of the anterior skeleton. A surprising result from Rodriguez-Esteban *et al.*² is that misexpression of Tbx4 or Tbx5 in chicks leads to

shortened limbs, loss of anterior structures, and disruption of the apical ectodermal ridge (an epithelial structure necessary for limb growth and patterning). This occurs even when excess Tbx4 is expressed in its usual domain, the hindlimb. In other words, too little or too much Tbx can inhibit development of skeletal elements. Again, animal models lacking Tbx5 in the limb should help to clarify the mechanism behind the defects seen in people with Holt–Oram syndrome.

The Tbx and Pitx proteins are transcription factors, so what are their targets? Although Tbx4 and Tbx5 are highly related, the specific defects associated with their misexpression indicate that they have distinct targets. Potential targets (either direct or indirect) include genes of the Hox homeodomain cluster. Takeuchi *et al.*¹ and Rodriguez-Esteban *et al.*² find that misexpression of Tbx4 in the wing induces expression of the hindlimb-specific Hox genes (*Hoxc9–Hoxc11*), and suppresses the forelimb-specific *Hoxd9*. Misexpression of Tbx5 has the opposite effect¹. The Tbx and Pitx proteins are also targets of each other — for example, Tbx4 expression in the hindlimb is activated by Pitx1 but repressed by Tbx5, and Tbx4 can regulate its own expression in the flank. Moreover, the Tbx proteins can bind to DNA either as dimers or as monomers⁹. So, misexpression of the Tbx proteins may respecify limb identity either by the binding of homodimers or monomers to inappropriate targets, or by the formation of aberrant heterodimers with altered or impaired ability to activate target genes.

The new results^{1–4} are our first real

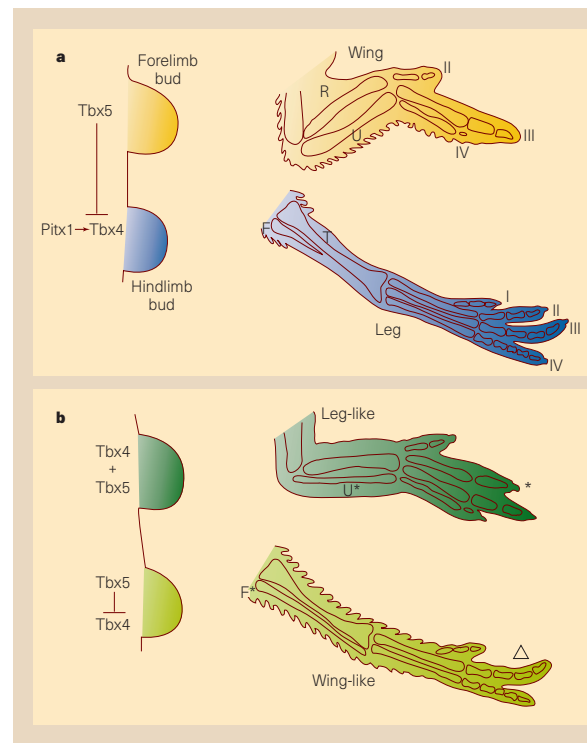


Figure 1 Effect of the Tbx and Pitx proteins on fore- and hindlimb identity. a, Expression of Tbx5 in the chick embryonic forelimb bud correlates with development of a wing. Tbx4 is restricted to the embryonic hindlimb bud through activation by Pitx1 and repression by Tbx5, allowing the development of a leg. b, New results^{1–4}, including those of Takeuchi *et al.*¹ and Rodriguez-Esteban *et al.*², show that misexpression of Tbx4 in the forelimb bud leads to the development of a leg-like structure. Misexpression of Tbx5 in the hindlimb bud, by contrast, leads to repression of Tbx4 and development of a wing-like structure. R, radius; U, ulna; F, fibula; T, tibia. Numerals indicate the digit pattern and asterisks indicate alterations in morphology. Δ indicates loss of a digit.



100 YEARS AGO

Mr A. Hall, of Highbury, has designed an almanac with the object of eliminating the inconvenience consequent on the various days of the months falling on different week days, owing to the changing number of days in each month. His scheme is to make New Year's Day separate from the rest, calling it January 0, and then divide the remaining 364 days into thirteen months of twenty-eight days each. Following this plan, therefore, any particular day of the month will always fall on the same day of the week, and this would, of course, be convenient for many purposes. The extra month he proposes to denote by the name "Christember." The almanac sent us is printed on this principle, and a useful item included is the table of corresponding dates between the Gregorian, Julian, Jewish and Mohammedan calendars. From *Nature* 27 April 1899.

50 YEARS AGO

During Easter 1947, an inspection was made of the seaweed cast up on the west mainland of Orkney. One heavy cast, 3 ft. thick at the water's edge, was found in a sandy bay north of the graveyard northwest of Stromness ... There is little chance of anything remaining intact before being cast ashore as the Atlantic waters break on this coast. The cast weed on this occasion, composed mainly of the fronds of *Laminaria Cloustoni*, had acted as a buffer for several large shells, such as *Pecten*, which were found intact. Normally, such shells become so broken up that they merely add to the shell sand comprising the foreshore in this region. From among the cast weed a bivalve, 4 in. long, was picked up which when discovered had its two halves hinged; the contents were absent. It has now been identified as *Tellina magna*, a native of the East American coast, North Carolina to West Indies – and is, I am informed, the first recorded instance of a marine invertebrate from the American continent to be found in British waters. Subsequent visits to the same area have produced only limpet shells and one small bivalve. There can be no doubt the cast weed was responsible for bringing the shell ashore intact. How the animal (or its ancestors) crossed the Atlantic can only be speculation.

From *Nature* 30 April 1949.

insight into the molecules that define and distinguish the identity of fore- and hindlimbs. But many questions remain. How is restricted expression of the *Pitx* and *Tbx* genes controlled? How do these genes specify the distinct bone, tendon and muscle structure of each limb? What role did these genes or their ancestors play in the development of limb types during evolution? The rapid identification of these genes, their targets and their phylogenetic relatives suggests that these gaps in our understanding will be filled quickly. □

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Microbial ecology

Molecular probing of deep secrets

Roger Summons

Techniques from molecular biology and organic geochemistry have been combined to provide a new tool for microbial ecologists, as shown by ribosomal RNA surveys and carbon isotopic analysis of sedimentary lipids reported by Hinrichs *et al.* on page 802 of this issue¹. Several lines of evidence suggest that methane gas seeping from unstable methane hydrates supports a newly discovered microbial community, which is unusual both in its genetic relationships and in its metabolism.

The concept of microbial diversity has been transformed by the growth in sequence data from ribosomal RNA (16S rRNA)². The cloning and sequencing of RNA from microbes living in their natural environments has revealed a genetic diversity beyond the dreams of researchers whose tools were limited to microscopy and cell culturing^{3,4}. RNA molecular probes have revealed previously unknown evolutionary lineages, as well as associations between the genetic structure of communities and their ecophysiology⁵.

As Hinrichs and co-workers¹ have shown, 16S rRNA cloned from shallow sediment samples above unstable methane hydrate deposits in the Eel River basin, offshore California, is dominated by a variety of previously unknown genes from archaea, which along with bacteria and eukaryotes comprise the three domains of life on Earth. These were accompanied by sequences from known anaerobes, including sulphate-reducing and Gram-positive bacteria. There were two main types of archaea discovered in the seep sediments, with some clones closely related to known methanogens (anaerobic methane-producing bacteria) of the order Methanosarcinales. Most sequences, however, were from a new and closely similar group that was distinct from, but related to, the methanogenic orders Methanomicrobiales and Methanosarcinales. Besides being distinguishable from known methanogens, these new

organisms also had sequences unlike any cloned from contemporary freshwater and marine environments.

As well as being new to science, these organisms have created an unusual sediment chemistry. Analyses of the membrane lipid and stable carbon isotope distributions of the samples tell us not only about their history, but also about their carbon metabolism. Methane is renowned for containing less ¹³C than virtually any other biological product on Earth, and large amounts of it are produced where organic matter is buried. Organic matter in the Eel River basin samples had less ¹³C than nearby controls, and methane gas bubbling into the water column had more ¹³C than near-surface methane hydrates in the same region. This is a powerful indicator that some members of the microbial community were consuming part of the methane from the decomposing hydrate.

Confirmation came from the carbon isotope contents of individual lipids present in the samples. Archaea have a diagnostic membrane component, a glycerol ether lipid known as 'archaeol'. Some members of the Methanosarcinales also have a unique variant of this lipid, *Sn*-2-hydroxyarchaeol. Both of these compounds were present in the seep samples, but absent from controls, and so depleted in ¹³C that they could only be the products of methane-consuming archaea. So, we have the enigma of organisms living on a diet of methane that have the same membrane lipids as organisms normally associated with methane production. Hinrichs *et al.*¹ hypothesize that cells containing the ¹³C-depleted lipids and the newly discovered 16S rRNA genes are one and the same.

Little is known about the specific metabolic processes of the microbes found in the Eel River basin and other methane-rich sediments. Carbon isotope and biomarker lipid patterns are quite different from those that characterize 'conventional' aerobic methane

consumption, and which have been measured in mussel communities at the surface of methane seeps⁶. Given that the sediments are anaerobic, and that there are sulphate-reducing bacteria present, it is virtually certain that methane is being oxidized at the expense of sulphate. Independent studies using radiotracers, stable isotopes and mass-balance analyses, point to a consortium of methanogens (somehow operating in reverse) and sulphate-reducing bacteria, which together are responsible for anaerobic methane oxidation⁷. However, until now, specific information about their genetic relationships has been lacking. The hypothesis favoured by Hinrichs *et al.* is that the new archaea are not simply methanogens operating in reverse, but a new group for which methane consumption is the predominant, or even exclusive, metabolism. If this is proven, it will have far-reaching consequences for our understanding of the physiology and evolution of archaea as well as their role in the carbon cycle.

Research conducted independently by Volker Thiel and co-workers⁸ has shown that distinctive limestones, the so-called 'calcarei *Lucina*' that are widely distributed in the Apennines, carry a similar geochemical signature for methane venting. These Miocene-age carbonates, in places packed with the remains of tubeworms, are highly depleted in ¹³C, with intracrystalline bio-

marker lipids for sulphate-reducing bacteria having even less ¹³C content. Moreover, biomarkers for archaea are among the most ¹³C-depleted yet reported. So, there is a neat alignment of geochemical patterns for a modern seep site and a 20 million-year-old counterpart.

These papers are exciting in the way they chart a new direction for biogeochemists. Studies of biogeochemical processing in contemporary environments, and particularly within the deep biosphere, can now be undertaken with more certainty about the ecology and physiology of the microbes. The new molecular probes that Hinrichs and colleagues have developed may soon be applied in a more quantitative manner. This will greatly strengthen our understanding of lipid biomarkers and their relationships to precursor organisms. These relationships were previously established indirectly, and sometimes haphazardly, from lipid analyses of organisms in cultures and corresponding samples from natural environments. Because the majority of rRNA genes cloned from natural environments are new to science, there is an obvious knowledge gap. As this is rectified and linked to compound-specific isotopic analyses of characteristic biomarkers, not just with carbon, but also with nitrogen, hydrogen, oxygen and sulphur, we have a key to improved information about biogeochemical processing. The

work of Thiel *et al.*⁸ shows how this type of knowledge can be used to project back in time.

Through various lines of investigation, anaerobic methane oxidation has been demonstrated as a viable metabolism for the deep biosphere and can be added to the compendium of ways in which microbes manipulate the distribution of chemical elements on a global scale. These microbes may eventually be cultured and their metabolic processes opened to further study. The combined organic-geochemical and molecular-biological strategy used by Hinrichs *et al.* is an important development in the study of global biogeochemical cycles. □

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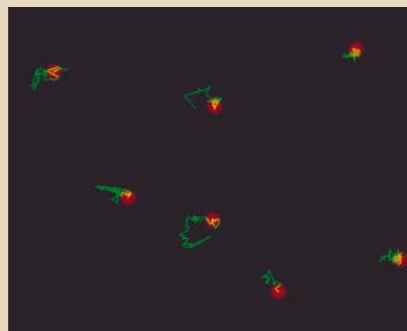
Cell biology

Coated-pit dynamics

Endocytosis — the process by which cell-surface receptors and other membrane proteins are taken up into the cell — occurs mainly through coated pits. These pits are specific sites on the plasma membrane where clathrin and the adapter protein AP-2 associate with the cargo. The pit invaginates, then a clathrin-coated vesicle is pinched off. Despite extensive characterization, we do not know whether these coated pits form randomly or at specific sites on the plasma membrane; how long they persist before detaching as coated vesicles; and how quickly coated vesicles uncoat.

Reporting in the first issue of *Nature Cell Biology* (1, 1–7; 1999), Gaidarov and colleagues now provide an insight into these questions. They looked at the formation and internalization of coated pits in living cells, using a fusion protein of green fluorescent protein (GFP) and the clathrin light-chain. Their work, which reveals an important relationship between the structural organization of clathrin-coated pits and the membrane cytoskeleton, has implications for organization of the plasma membrane.

The authors found that coated pits



labelled with GFP-clathrin appear gradually, persist for several seconds, then disappear abruptly without moving far from where they originated. That is, detached coated vesicles are stripped of their clathrin coat in the vicinity of the pit — they do not move through the cytoplasm first. Surprisingly, Gaidarov *et al.* saw that the coated pits and vesicles do not move outside regions of about 0.5–0.8 μm in diameter (see picture). But latrunculin B — which inhibits actin assembly — relaxes this restricted mobility, implying that an actin-based framework maintains the structural organization of clathrin-coated pits.

The authors found that the formation

of coated pits also seems to be coordinated by an underlying membrane skeleton. Coated pits appeared and disappeared at defined, rather than random, sites on the plasma membrane. Indeed, once fluorescence at a coated pit disappeared, it usually reappeared at the same site. This meant that coated pits did not form at all over large areas of the membrane.

These observations are difficult to reconcile with the conventional view of how coated pits are formed. Diffusible transmembrane receptors or membrane-docking sites are thought to recruit AP-2 and clathrin to form a coated pit. But the new data indicate that coated-pit formation is coupled to events at the membrane skeleton, possibly through scaffold proteins found in specific (and limited) places. Future work with GFP fusion proteins should resolve this discrepancy, allowing us to see how coated pits form and work in a living cell.

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Climate change

Linear and nonlinear signatures

Klaus Hasselmann

It is probable that the observed warming of the Earth by 0.7°C over the past 100 years is caused by anthropogenic emissions of greenhouse gases, and that if trends continue the Earth will warm by another $2\text{--}3^{\circ}\text{C}$ before the end of the next century¹. But can claimed increases in the frequency of extreme events such as storms, floods and droughts be ascribed to human influence, or are they simply due to the natural vagaries of climate? And what might be the effects of possible anthropogenic climate change on the occurrence of such anomalies in the future?

It is the changes in the statistics of extreme events, rather than in the mean temperature, that will probably have the strongest impact on human living conditions. On page 799 of this issue², Corti *et al.* tackle this statistical question. They suggest that there is a direct interconnection between short-term climate variations on timescales of less than five years, which are known to have a strong influence on extreme weather events, and mean climate change on longer timescales, such as a change in the mean temperature or pressure distribution.

The importance of their study lies in their bringing nonlinear analysis to bear on the problem. They argue that although climate undergoes continual fluctuations, it nevertheless has a tendency to linger in preferred states, or attractors, characterized by large-scale anomaly patterns. These patterns can be related to well-known phenomena, such as El Niño, the North Atlantic Oscillation or the Pacific North American anomaly, which have been widely studied in the context of short-term climate variability. Climate change on longer timescales can be largely explained as changes in the frequency of occurrence of these dominant climate states, while the states themselves remain unmodified^{3–6}. The principle applies to both natural, longer-term climate variations, and anthropogenically generated climate change.

The hypothesis is extremely simple. But it has major implications for our understanding of longer-term climate variability, and is also relevant to the detection of anthropogenic climate change (which involves distinguishing between natural and anthropogenic change on these timescales¹).

To appreciate the new perspective of Corti *et al.*, it should be recalled that externally forced climate change over long timescales has been traditionally treated as a linear response problem. The forcing is an applied perturbation, and can be natural (for instance, changes in solar insolation or volcanic activity) or anthropogenic (increasing CO_2 in the atmosphere). The linearization is

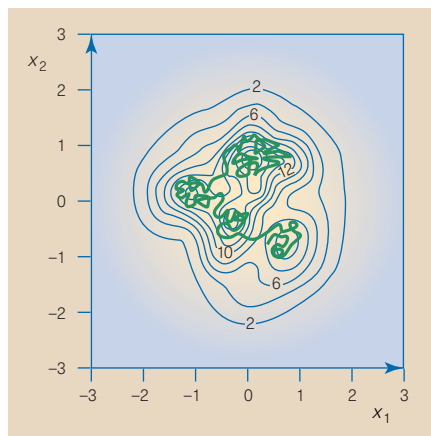


Figure 1 Random walks and climate change. Shown here is the random-walk trajectory of a stochastically forced particle wandering between potential wells; the process corresponds to the probability distributions shown in Fig. 1 of the paper by Corti *et al.*² (page 800). The particle coordinates x_1, x_2 correspond to the amplitudes of the first two empirical orthogonal functions. The mean climate anomaly pattern is given by the mean particle position $\langle x \rangle$.

valid for any system, linear or nonlinear, time-independent or time-dependent. For fluctuating systems, it can be applied to the time average, and it is also appropriate for chaotic systems with time-independent statistics.

The provisos for applying the linear approach are that the forcing must be small and that the system must not be close to a bifurcation point (for example, in the case of climate, to a collapse of the deep-ocean circulation). For the present discussion, this possibility can be excluded.

Numerical experiments show that the linear approximation is acceptable for climate change thought to be generated by current anthropogenic forcings. The cross-frequency interdependence proposed by Corti *et al.* is, by contrast, fundamentally nonlinear. But their analysis complements rather than contradicts the traditional approach by providing deeper insight into the nonlinear dynamics underlying the linear response.

Their concept is best explained in terms of a simple, stochastically forced potential-well model, which is perhaps more familiar to readers not versed in chaos theory than the three-component Lorenz model that the authors themselves invoke (it also captures more of the stochasticity inherent in the climate system's many degrees of freedom). In analogy with Brownian motion, stochastic climate models explain the low-frequency

variability of climate as the response of the slowly responding components of the climate system (the oceans, biosphere, ice caps and so on) to random forcing by rapid fluctuations in atmospheric weather⁷. In many applications, it is further assumed that the dynamics of the 'slow' climate system can be treated as linear^{8,9}.

Corti *et al.* base their hypothesis on the analysis of 45 years of monthly-mean atmospheric pressure fields. They reduce the dimensions of the original data set to two by projecting the data onto the first two principal-component patterns (empirical orthogonal functions) of the short-term climate variations. They then investigate the two-dimensional probability distributions of the coefficients x_1, x_2 of these patterns for the complete time series, including both short and long timescales.

A linearized stochastic forcing model of Corti and colleagues' two-component subsystem would correspond to the random-walk $x_1(t), x_2(t)$ of a stochastically forced particle trapped in a parabolic potential well with a minimum at the origin. This would yield a Gaussian (Maxwellian) probability distribution with a maximum at the origin, but it is not what the data show. Rather, Corti *et al.* find a number of well-separated probability maxima; this corresponds to a randomly forced particle wandering between a number of potential wells (Fig. 1).

How will such a particle respond to external forcing? An external force F changes the relative level of the potential wells. This modifies the 'tunnelling' probability of a particle traversing the potential ridges separating the potential wells, and thereby the average residence times within the different potential wells. For a small forcing, the change $\langle \delta x \rangle$ in the mean position of the particle (that is, the mean climate response pattern) is proportional to the forcing, in accordance with the linear response model, $\langle \delta x \rangle = TF$. However, the response matrix T is determined by the changed relative weightings of the basic variability patterns, which are governed by the nonlinear dynamics underlying the potential-well structure.

The external forcing could be either anthropogenic or natural. Or it could be 'pseudo-external', representing internal climate interactions on long timescales. For the detection of a signal of anthropogenically induced climate change, it will be necessary to determine the different effects of these forcings on the relative probabilities of the basic modes of climate variability.

The model of Corti *et al.* is still rudimentary. For instance, the relevant mode exhibiting low frequency in its probability distribution contained less than 13% of the total variance. Nonetheless, the basic concept is attractive and should be pursued further — for example through the construction of simple 'principal interaction pattern'

models^{10–13} to test the nonlinear dynamics underlying the authors' hypothesis. □

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Apoptosis

Life and death in a FLASH

Jan Paul Medema

In some ways, a cell is a bit like a spy. Many spies, at some point in their career, are found out (and have to swallow a cyanide pill), or they may defect and face execution by former colleagues. Likewise, every cell in a multicellular organism can commit suicide (in situations of extreme stress, for instance), or it may be killed when surrounding cells activate specific cell-surface receptors. The basic mechanism of cellular suicide is similar in organisms ranging from the nematode worm to humans. And on page 777 of this issue, Imai *et al.*¹ describe a newly discovered — and completely unexpected — mammalian protein that is involved in it.

Cellular suicide, known as programmed cell death or apoptosis, is a tightly controlled mechanism. In the nematode *Caenorhabditis elegans*, it is dictated by three proteins². CED-3, the 'executioner', belongs to a family of killer proteases known as the caspases. It is activated by interaction with CED-4, but inhibited by CED-9. A similar trinity has

been identified in mammals³. Here, Apaf-1 (equivalent to the nematode CED-4), binds to and activates caspase-9, one of the many mammalian CED-3 homologues. Apaf-1 must itself be activated, and this occurs through a conformational change induced by the binding of ATP and cytochrome *c*. Activated Apaf-1 molecules join together (oligomerize) and, along with the associated caspase-9, form a protein complex dubbed the apoptosome. This provides a platform for caspase-9 activation. The third component of the mammalian trinity is the CED-9 homologue, encoded by members of the Bcl-2 family. These can block activation of caspase-9 by inhibiting the release of cytochrome *c* or, possibly, by interacting directly with Apaf-1.

But mammalian cell death has an additional feature that has not been identified in nematodes. Mammalian cells express death receptors — such as the well-known CD95 (APO-1/Fas) — on their surface. When these

receptors oligomerize, they induce apoptosis. Propagation of the death signal requires an interaction between the intracellular tail of CD95 and the so-called death domain of an adapter molecule called FADD/MORT1. The other half of FADD/MORT1 constitutes a 'death-effector domain' (DED), which is thought to interact with the DED of caspase-8 (another CED-3-like molecule), to form the 'death-induced signalling complex'. Close physical interaction of several caspase-8 molecules within this complex is thought to activate the caspases and, hence, switch on intracellular suicide.

Imai and colleagues¹ now report that this scheme is oversimplified. They have identified a protein called FLASH, which interacts with the DED of caspase-8. The carboxy-terminal region of FLASH contains a DED-like domain, which is responsible for the interaction with caspase-8. FLASH also has a region of homology to CED-4/Apaf-1, through which FLASH molecules seem to associate with each other. The authors suggest that FLASH is involved in CD95-induced apoptosis (Fig. 1). First, cross-linking studies show that FLASH is recruited to the CD95 receptor in the cell. Second, CD95-induced apoptosis is facilitated by overexpression of FLASH. And third, FLASH mutants (which encode only the DED-like or CED-4-like region) inhibit apoptosis in a dominant-negative fashion.

So, then, could FLASH be a CED-4/Apaf-1-like protein that regulates CD95-induced activation of caspase-8? To many it will come as a surprise that CD95-mediated activation requires such a molecule, yet there are unmistakable parallels between FLASH and Apaf-1/CED-4: each of them can self-associate through the 'CED-4-like' domain; each interacts specifically with its caspase and can bind ATP; and each also interacts with a member of the Bcl-2 family. In the case of FLASH, Imai *et al.* have found this factor to be the adenoviral protein E1B19K, which inhibits CD95-induced apoptosis specifically at the level of caspase-8 activation⁴. It is, therefore, tempting to speculate that FLASH is a player in a CED-9/CED-4/CED-3-like regulatory structure.

Some gaps remain. First, the huge amino-terminal half of FLASH probably has a function, yet deleting it has no apparent effect. Second, it is unlikely that there is any functional consequence when ATP binds to FLASH, because CD95-induced activation of caspase-8 does not depend on the energy status of a cell⁵. Third, although E1B19K binding may affect FLASH during viral infection, the CED-9-like regulation probably involves a member of the mammalian Bcl-2 family in uninfected cells. But no Bcl-2 protein has yet been shown to modulate caspase-8 activation at the level of the receptor — and, thus, at the level of FLASH.

Irrespective of whether FLASH is a CED-

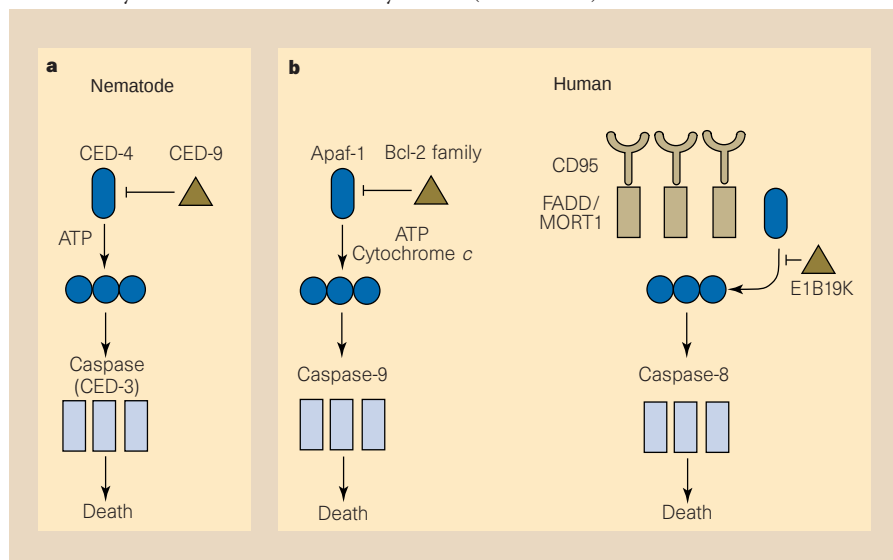


Figure 1 Apoptotic pathways in the nematode and in mammals. a, In the nematode, the killer protease (or caspase; CED-3) is activated by CED-4 but inhibited by CED-9. b, In mammals there are many caspases, one of which is activated by Apaf-1. Members of the Bcl-2 family inhibit apoptosis. There is also a receptor-activated pathway, which is activated by signals arriving at the CD95 receptor. Imai *et al.*¹ have discovered that FLASH interacts with an adapter molecule, FADD/MORT1, which binds CD95, and also with the caspase. FLASH could control apoptosis at the level of caspase activation.

4 homologue, the biggest challenge now is to work out how — and when — it regulates apoptosis. One hypothesis is that FLASH, much like Apaf-1/CED-4, simply facilitates cell death. Once FLASH has been recruited to the receptor it could, by virtue of its ability to self-associate, assemble several caspase-8 molecules and provide a platform for their activation. Modulation of caspase-8 activation is known to determine the fate of a cell. In fibroblasts, for example, the apoptosis-promoting *c-myc* oncogene is thought to increase caspase-8 activation⁶. Conversely, c-FLIP, which inhibits CD95-induced apoptosis *in vivo*, does so by blocking this activation⁷. Although Imai *et al.*¹ detected a direct interaction between c-FLIP and FLASH, the role of FLASH in modulating caspase-8 is not yet known.

Finally, cells can be classified as type I or type II depending, in part, on how efficiently they form a death-induced signalling complex⁸. Imai and colleagues did not detect any difference between the levels of FLASH expression in these two types of cell. However, other mechanisms for altering the activity

of FLASH — such as sequestration or phosphorylation — cannot be excluded. This is, in fact, an essential point, because the death of activated peripheral T cells is suggested to involve a switch from type I to type II⁹. FLASH is an attractive candidate for controlling this cellular suicide as well, because it may dictate execution by 'former colleagues'. It shouldn't be long before we find out whether this is the case. □

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Cell cycle

Mitotic treasures in the nucleolus

Jeffrey B. Bachant and Stephen J. Elledge

It is not often that one gets the opportunity to solve a basic problem and discover a regulatory principle in the process. But this is precisely what is reported by Amon and co-workers¹ on page 818 of this issue, and by Shuo *et al.*² in *Cell*. They were investigating how the cell regulates exit from mitosis — the final, chromosome-segregation phase of the cell cycle — and re-entry

into the first phase, G1. And they have found that the key is sequestration of a protein phosphatase called Cdc14 in the nucleolus.

When cells enter mitosis, a series of carefully orchestrated, irreversible events occurs (reviewed by Morgan³). Depending on the organism, this includes breakdown of the nuclear envelope, condensation of chro-

mosomes, assembly of the mitotic spindle, attachment of chromosomes to the spindle and, ultimately, segregation of the chromosomes to opposite poles of the cell (anaphase). After mitosis, the cell must be cleaved in two, then the two daughter cells return to a state (known as interphase) in which they are prepared for the next round of DNA synthesis and cell duplication. The main block to this process is the activity of a complex containing cyclin B and the cyclin-dependent kinase-1 (Cdk1), which both promotes entry into mitosis and prevents exit from it.

Cells circumvent this block by inactivating mitotic kinases such as cyclin B/Cdk1. In the budding yeast *Saccharomyces cerevisiae*, this is done by two overlapping mechanisms: destruction of cyclin B (or Clb in yeast), which is catalysed by the so-called anaphase-promoting complex (APC); and binding of a CDK inhibitor called Sic1 to the cyclin/Cdk1 complex. After cells have segregated their chromosomes, Sic1 accumulates and the APC becomes activated through association with an activating subunit termed Cdh1 (also known as Hct1)^{4–6}. During mitosis, Cdh1 is phosphorylated — and inhibited — by cyclin B/Cdk1. It is activated only when a phosphatase called Cdc14 removes the inhibitory phosphate group^{7,8}.

How does Cdc14 turn the tables on cyclin B/Cdk1? First, it dephosphorylates (and, hence, activates) a transcription factor called Swi5, allowing fresh Sic1 to be transcribed (Fig. 1). This newly produced Sic1 is immediately targeted for degradation by Cdk1 phosphorylation^{9,10}, so the next effect of Cdc14 is to dephosphorylate Sic1 and reduce Cdk1 activity¹¹, thereby allowing Sic1 to accumulate. Finally, as mentioned above, Cdc14 dephosphorylates Cdh1, allowing it

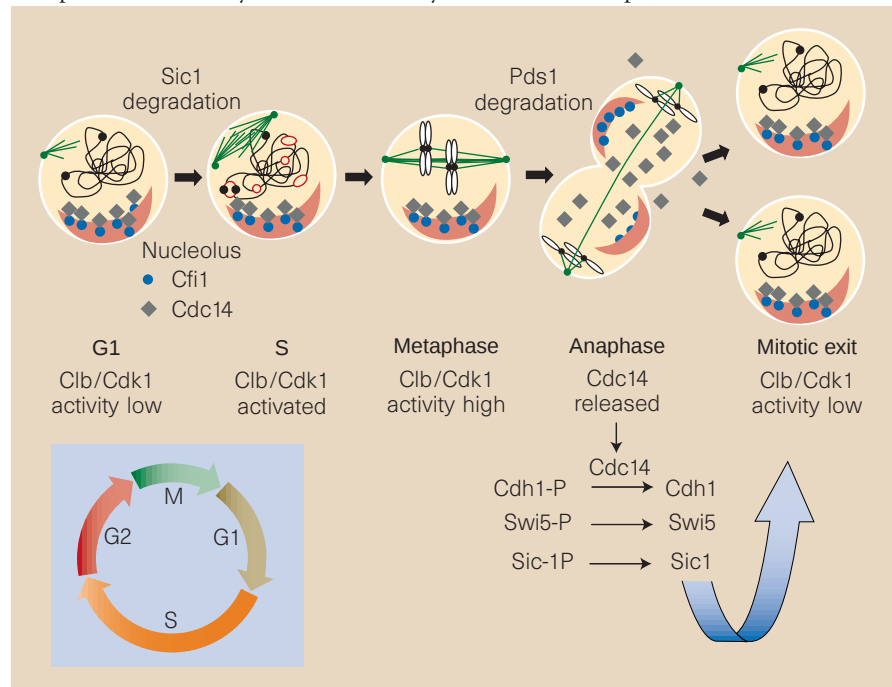


Figure 1 Model for regulation of Cdc14 during the cell cycle. Amon and colleagues¹ and Shuo *et al.*² have found that, in G1, inactive Cdc14 is sequestered in the nucleolus through its association with the inhibitory factor Cfi1. After degradation of the cyclin-dependent kinase (CDK) inhibitor Sic1, mitotic forms of Cdk1 accumulate. This results in DNA synthesis, entry into mitosis, and activation of the anaphase-promoting complex (APC). The APC catalyses destruction of the anaphase inhibitor Pds1 to initiate anaphase¹³, and is also proposed to initiate a signal-transduction cascade that liberates Cdc14 from the nucleolus. Active Cdc14 facilitates inactivation of mitotic Cdk1 by activating Cdh1 and promoting accumulation of Sic1. Cdk1 inactivation then promotes cell division, breakdown of the spindle and return to interphase. Phases of the cell cycle are shown in the inset: G1, pre DNA synthesis; S, DNA synthesis; G2, period between DNA synthesis and mitosis; M, mitosis, followed by cell division.

to bind and activate the APC. So, Cdc14 can be viewed as an 'anti-Cdk1' phosphatase, directly reversing Cdk1's phosphorylation of key targets. Once Sic1 has accumulated and the APC is activated by Cdh1, cells can inactivate their mitotic CDKs and exit mitosis.

Amon and colleagues¹ and Shou *et al.*² have now tackled the problem of how Cdc14 is controlled. They began by investigating where Cdc14 is found during the cell cycle, and, using immunolocalization, made the remarkable discovery that Cdc14 is dramatically relocalized within the nucleus. During the G1 and S phases, Cdc14 is found in the nucleolus — a sub-compartment within the nucleus. Then, just as the cells enter anaphase, Cdc14 spreads to the entire nucleus and also, to some extent, the cytoplasm. This is the first report of regulated localization to the nucleolus as a regulatory mechanism, and the authors suggest that Cdc14 is activated by release from nucleolar sequestration, allowing it to associate with its substrates.

This is a beautiful hypothesis. But the great tragedy of science, as noted by Thomas Huxley, is the slaying of a beautiful hypothesis by an ugly fact. So, in this case, are the supporting facts as attractive as the model? To find out, the two groups looked at what might anchor Cdc14 to the nucleolus. Using a protein-interaction screen they found that Cdc14 binds to a protein called Cfi1 (also known as Net1), the localization of which precisely overlaps with the pattern of Cdc14 in the nucleolus. Unlike Cdc14, however, Cfi1 remains in the nucleolus during anaphase. Cfi1 shares a stretch of sequence with a known phosphatase regulator called Reg1, indicating that it might, perhaps, regulate Cdc14 (ref. 1).

Genetic results indicate that, in fact, Cfi1 inhibits Cdc14, possibly by directly affecting its phosphatase activity. The authors first reasoned that, if Cfi1 is the anchor, when it is removed from cells Cdc14 should be released. And this is just what they found — in *cfi1* deletion mutants, Cdc14 was no longer localized to the nucleolus^{1,2}. Amon and colleagues further found that the *cfi1* mutants behaved like cells overproducing Cdc14. Such cells have problems entering S-phase because, in order for them to do so, Sic1 must be phosphorylated and destroyed. But the overproduced Cdc14 partially reverses this phosphorylation, delaying destruction of Sic1. Overproduction of Cfi1, by contrast, is lethal. Cells arrest late in mitosis and have elongated spindles — similar to *cdc14* mutants. The toxic effects of Cdc14 overproduction can be overcome by simultaneously overproducing Cfi1 (ref. 1).

Amon and colleagues also found that when Cfi1 is overproduced in normal cells, Cdc14 is not sequestered in the nucleolus; instead, it is found with Cfi1 throughout the cell. This indicates that, when levels of Cfi1

are high, its binding sites in the nucleolus become saturated and it can inhibit the dispersed Cdc14 as well. Shou *et al.*² found that recombinant Cfi1 could reduce Cdc14's phosphatase activity *in vitro*. So, Cfi1 may be a multifunctional inhibitor, working both by sequestering Cdc14 in the nucleolus and by interfering with it biochemically.

But why do cells use nucleolar sequestration — as opposed to the commonly observed cytoplasmic localization — to regulate Cdc14? Perhaps Cdc14 has substrates in both the cytoplasm and the nucleus, leaving the nucleolus as the only place where it does not normally function. Whatever the reason, nucleolar sequestration is likely to be a general regulatory mechanism. In fact, reporting in *Nature Cell Biology*, Weber *et al.*¹² show that the tumour-suppressor protein p19^{Arf} activates p53 by sequestering a p53 inhibitor, Mdm2, in the nucleolus. Arf mutants that bind Mdm2 but do not localize to the nucleolus are biologically inactive. Although there is no evidence that this is regulated sequestration, it is clearly a similar mechanism. So, the nucleolus, which has always been viewed as a factory for the production of ribosomes, is now taking on the appearance of a resort — a nice, quiet place to visit and get away from it all. □

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April fool The News and Views article "Millennium bug" by R. S. Siew — published on 1 April (*Nature* **398**, 376; 1999) — was, we can confirm, an April fool. Most of the references, such as the citation of Sisir, C. and Linnellim, K. *Silico* (in press) on the crucial Y2K mutation, are figments of the author's backward thinking. G. K. Chesterton, cited as having published a paper in *Protista* in 1997, in fact died in 1936, and as far as we are aware did no research in this field. □

Daedalus

Theological chemistry

The most convincing evidence for religious belief is subjective. Many people claim to sense the presence of God, to be able to communicate with Him in prayer, or receive comfort from Him in trouble. But to others, praying simply feels like talking into a dead telephone. Even devout believers sometimes suffer 'the dark night of the soul' when the divine presence cannot be sensed.

One theory is that the religious sense is chemical. Many primitive religions use psychotropic drugs and hallucinogens in their rituals. Nitrous oxide, ether and LSD have also been claimed to open the user's mind to higher reality. Daedalus disagrees. Such intoxicants, he reckons, merely stir up noise and nonsense inside the brain. He wants to get past the 'earthquake, wind and fire' to reach the 'still, small voice' of the authentic spiritual experience.

So he plans to conduct brain scans on monks and nuns at prayer, to identify the active region of the brain. Successful prayers and 'dark night' failures should show different patterns. With very good luck, an NMR scan might even be able to identify the molecule metabolized in a successful religious experience.

Another way of identifying it depends on Daedalus's theory of last week, that the spirit world shares the 3 K temperature of the cosmic microwave background, and that spiritually important molecules radiate spontaneously into that world. The black-body peak at 3 K is at 310 GHz, a frequency band in which molecular rotational and librational resonances occur. Isotopically substituted molecules with shifted resonances should therefore be spiritually less effective. By synthesizing candidate substances enriched with ²H, ¹³C or ¹⁵N, and injecting them into the test monks and nuns, the crucial religious metabolite could be identified. People in whom it is richly present will be believers, those without it will be hard-boiled materialists. A simple tablet or injection will then enable the latter to feel religious experience for themselves.

Daedalus's 'Theological Prozac' will at last open the private, subjective claims of religion and mysticism to scientific study. It will make spiritual experiences freely accessible and reproducible, allowing them to be classified and their implications understood. With luck, the resulting illumination will bestow spiritual comfort on the users, unaccompanied by the stern orthodox convictions attached to it by the more doctrinal aspects of religion.

David Jones

Acoustic guide in bat-pollinated flower

Several hundred species of neotropical plants are pollinated by glossophagine bats^{1,2}. These bats use their highly developed sonar system for orientation, so we might expect bat-pollinated flowers to have evolved acoustically conspicuous structures to make them easier to detect. We find that the bat-pollinated neotropical vine *Mucuna holtonii* directs its echolocating pollinators to its flowers by means of an acoustic nectar guide. The flower contains a small concave 'mirror' that works like an optical cat's eye, but in the acoustic domain, reflecting most of the energy of the bats' echolocation calls back into the direction of incidence.

M. holtonii is found along the edges of rainforests or along creeks throughout Central America. Its flagelliferous inflorescences, which hang down several metres from the canopy, have 3–8 open flowers per

night during the flowering period. The flowers (about 4 cm in length) are of the papilionaceous type (Fig. 1), with two petals constituting the keel, two lateral petals forming the short 'wings', and a fifth, upper petal forming the triangular 'standard', or vexillum, which is raised when the bud comes into flower. Pollen is released explosively, in a manner unique among bat-pollinated flowers but typical of some Papilionaceae, when the flower is visited for the first time^{3,4}.

To reach the nectar, the bat lands on the flower and presses its snout into the slit between the wings. This causes the keel to burst and the staminal column, being under tension, catapults most of the pollen load onto the bat's rump. On the first visit the flower releases up to 100 μ l of nectar, but subsequent visits to an exploded flower will yield only 10–20 μ l of nectar. Nevertheless, the inflorescence remains attractive to bats as several flowers mature successively during the first half of the night, maturation being indicated by the raising of the vexillum. For efficient foraging, therefore, a bat should search for newly opened virgin flowers with raised vexilla.

We tested whether the raised vexillum could be a critical signal indicating the presence of a mature flower to the bats. From dusk until about 21:00, inflorescences were covered with plastic bags, preventing bats from exploding mature flowers, to maximize the number of virgin flowers available for manipulation. We then removed the vexillum from about half of the virgin flowers in each inflorescence, the other half being kept intact as controls. After two hours, we counted how many flowers were exploded, indicating that they had been visited by bats. Removal of the vexillum led to a remarkable reduction in the number of visits (21% of 203 flowers were visited compared with 88% of 321 intact flowers).

To exclude a possible role of visual or olfactory cues, we repeated the experiment using flowers in which a small pad of cotton wool was inserted into the cavity of the vexillum, altering the echo-reflecting properties of the vexillum while retaining the shape and possible sources of odour. Once again, very few manipulated flowers were exploded compared with intact flowers (17% of 172 flowers were visited compared with 66% of 143 intact flowers).

We examined the echoes reflected from flowers when exposed to artificial sound sweeps imitating the natural echolocation calls of *Glossophaga commissarisi*, the main pollinator of *M. holtonii* in the area⁵. A comparison of echoes produced by virgin flowers and those in which the vexillum had

been filled with pads of cotton wool revealed that the echo of the flower was dominated by the echo of the vexillum (Fig. 1). Despite the small size of the vexillum (about 19 mm across, which is about 5–6 times the wavelength of the echolocation calls), echoes reflected from an isolated vexillum mounted on a thin wire had an astonishingly high amplitude; the target strength (the sound pressure level of the echo compared with that of the signal when having travelled the same distance) was -15.5 ± 1.2 decibels.

The spectral composition of the echo was dependent on the angle of sound incidence, but the amplitude was high within a large cone of incidence angles (about $\pm 40^\circ$; Fig. 1). The echoes should therefore be acoustically conspicuous for a passing bat, which would receive high-amplitude echoes for many calls from the same spot, whereas leaves and other flat surfaces would reflect high-amplitude echoes for only one echolocation call.

For a structure to be easily detectable by foraging bats, echoes should reflect as much energy as possible back to the sound source. When we moved the microphone in an arc around the vexillum while keeping the sound source fixed, we found that most energy was reflected back in the direction of sound incidence, regardless of whether the vexillum faced the sound source or was rotated by 30° (Fig. 2), like a cat's eye or a triple mirror in the optical domain.

The idea that the concave geometry of

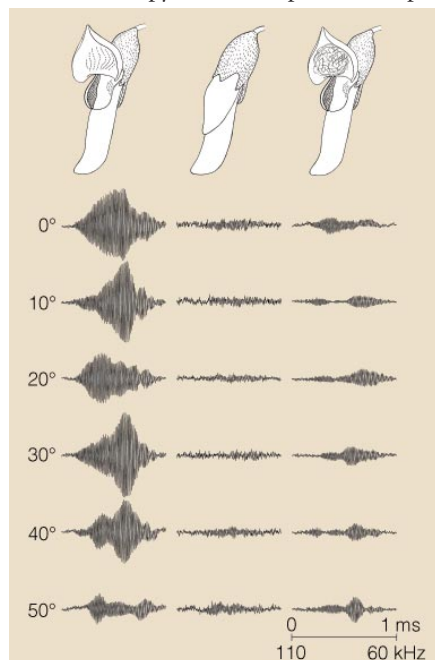


Figure 1 Echoes obtained from *Mucuna holtonii* flowers. Top, a virgin, intact flower (left), a bud (middle) and a flower in which the vexillum was filled with a pad of cotton wool (right). Echoes reflected from each of the flowers are shown underneath. The signal was a 1-ms sound sweep, the frequency of which was linearly modulated from 110 to 60 kHz, with a triangular envelope (0.5 ms rise and decay time). The flower was mounted on wire 20 cm from the loudspeaker, so the signal and the returning echo could not overlap. Echoes were picked up by a Bruel and Kjaer 1/8-inch microphone (B&K 4135, used without grid), mounted below the speaker (distance between centres, 2 cm); 256 recordings were averaged. The flower was rotated about the vertical axis to vary the angle of sound incidence. Experiments were carried out in lowland rainforest near the field station of the Organization for Tropical Studies at La Selva, Costa Rica.

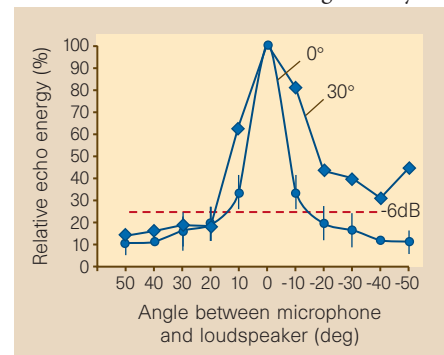


Figure 2 Sound energy reflected by the vexillum of *M. holtonii* when exposed to frequency-modulated sound sweeps. The loudspeaker was fixed at two positions: with the vexillum facing the sound source (0°), and with the vexillum rotated by 30° in the horizontal plane. The microphone was moved in the horizontal plane in an arc of radius 20 cm around the vexillum between -40° and $+40^\circ$. The sound stimulus was a 1-ms sweep with a rectangular envelope, modulated in frequency from 120 to 80 kHz. To calculate the reflected sound energy, the amplitude function of the echo was squared and integrated. In both mountings, most of the sound energy is reflected back in the direction of the loudspeaker.

the vexillum may be an adaptation to the echolocation system of glossophagine pollinators is supported by the fact that species of *Mucuna* that are visited by small palaeotropical Megachiropteran bats (which do not have an echolocating system) do not possess raised and concave vexilla^{6,7}. We suggest that similar adaptations may have been evolved by other neotropical bat-pollinated plant species.

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Cognitive restoration of reversed speech

Speech is the most complex auditory signal and requires the most processing¹. The human brain devotes large cortical areas^{2,3} to deciphering the information it contains, as well as parsing speech sounds produced simultaneously by several speakers⁴. The brain can also invoke corrective measures to restore distortions in speech⁵; for example, if a brief speech sound is replaced by an interfering sound that masks it, such as a cough, the listener perceives the missing speech as if the brain interpolates through the absent segment. We have studied the intelligibility of speech, and find it is resistant to time reversal of local segments of a spoken sentence, which has been described as “the most drastic form of time scale distortion”⁶.

We subdivided a digitized sentence into segments of fixed duration (say, 50 ms). Every segment was then time-reversed without smoothing the transition borders between the segments. The entire spoken sentence was therefore globally contiguous, but locally time-reversed, at every point (A + B in Fig. 1). Listeners report perfect intelligibility of the sentence for segment durations up to 50 ms, and partial intelligibility for segment durations exceeding 100 ms (Fig. 1, bottom), with 50% intelligibility occurring at about 130 ms; by psychoacoustic standards, such segment distortions are very long. Many defining features of speech sounds are rapid temporal transitions with durations well within the reversal window.

Perception of speech against local time

reversal is robust even if alternating segments are shifted in time (A + delayed B). Speech also remains intelligible if odd-numbered segments are displaced forwards in time by two or three times the duration of the window. For example, for segments of 100 ms, shifting the odd-numbered segment forward in time by 200 ms reduces the intelligibility rating by only 15%. For segments of 50 ms, intelligibility is not significantly affected by a displacement of 100 or 200 ms, but the speech does sound more echoic. Furthermore, the results are not changed if half the segments (A in Fig. 1) are presented to one ear and the other half (B in Fig. 1) to the other ear.

When subjects listen repeatedly to locally time-reversed sentences with moderately long windows (100 ms), they report that previously unintelligible words become clear. This type of ‘learning’ is not simply due to an improvement in identification, as subjects say they can now hear actual words, indicating some form of cognitive recalibration. The experience is similar to familiarization with a newly heard accent.

These findings lend support to recent theories^{7,8} of speech encoding that state, contrary to conventional thinking, that a detailed auditory analysis of the short-term acoustic spectrum is not essential to the speech code. Rather, the ultralow-frequency modulation envelopes in the order of 3 to 8 Hz are critical cues to intelligibility. Although the amplitude spectrum of a waveform is unaffected by time reversal, the temporal envelopes, as well as the fine structure of the running spectrum, are highly distorted for such sounds. The

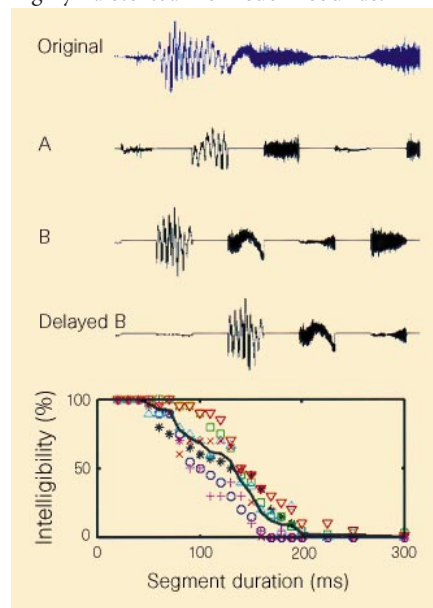


Figure 1 Segments of speech showing the effects of time reversal. Top, 50-ms segments of original and locally time-reversed (A + B) speech. Bottom, subjective intelligibility rating by seven subjects (different symbols). The solid line shows the average rating score.

advantage of a robust speech-encoding system that uses higher-order corrective measures and ultralow-frequency cues is obvious in noisy environments where the listener needs to extract perceptually and identify a stream of speech cues that compete with extraneous noise, as in the ‘cocktail party effect’⁹.

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Co-carcinogenic effect of β -carotene

Epidemiological and animal studies on vitamin A and its analogues support the hypothesis that β -carotene can prevent cancer in humans¹. However, chemoprevention trials have unexpectedly shown that β -carotene, either alone or in combination with vitamin A or vitamin E, actually increases lung-cancer incidence and mortality in heavy smokers and asbestos workers^{2–4}. We find that β -carotene in rat lung produces a powerful booster effect on phase I carcinogen-bioactivating enzymes, including activators of polycyclic aromatic hydrocarbons (PAHs), and that this induction is associated with the generation of oxidative stress. Our findings might explain why β -carotene supplementation increases the risk of lung cancer in smokers.

The α -tocopherol β -carotene (ATBC) and β -carotene retinol efficacy trial (CARET) chemoprevention studies suggested that heavy smokers should avoid high-dose β -carotene supplements because of an increased risk of lung cancer^{2–4}. The ATBC study (29,133 participants) recorded 18% more lung cancers and 8% more overall deaths in smokers taking β -carotene; in CARET (18,314 participants), there were 28% more lung cancers and 17% more deaths in smokers and asbestos workers who were taking β -carotene and vitamin A supplements. Rather than initiating new tumours, β -carotene might have co-carcinogenic properties on latent ones⁵.

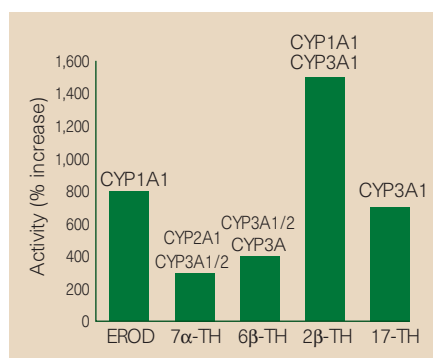


Figure 1 Enzymatic CYP induction by β-carotene. Male Sprague-Dawley rats (aged 6–7 weeks, 140 ± 10 g) maintained on a standard laboratory diet received by mouth daily 500 mg per kg body weight of β-carotene (Aldrich, Milan) for five consecutive days; controls received only corn oil. Because of species specificity, the purpose of the *in vivo* experiments was to find evidence of the co-carcinogenic potential of β-carotene⁷, not to mimic a trial situation. Rats were fasted for 16 h before being killed humanely in accordance with approved procedures. Ten rats were in each group; lung microsomes were tested immediately for ethoxyresorufin *O*-deethylase (EROD) and testosterone hydroxylase⁸ (TH).

Because β-carotene is known to be an anti-genotoxic agent⁶, we decided to investigate whether it might act by means of epigenetic mechanisms, such as those involving cytochrome P450 (CYP) changes⁷.

We found a highly significant increase in the carcinogen-metabolizing enzymes CYP1A1/2 (activating aromatic amines, polychlorinated biphenyls, dioxins and PAHs), CYP3A (activating aflatoxins, 1-nitropyrene and PAHs), CYP2B1 (activating olefins and halogenated hydrocarbons) and CYP2A (activating butadiene, esamethyl phosphoramidate and nitrosamines) in the lungs of rats supplemented with high doses of β-carotene. This was documented by marked increases in the following probes: ethoxyresorufin *O*-deethylase activity (CYP1A1-linked), testosterone 7α-hydroxylation (CYP1A1/2 and CYP2A1), 6β-hydroxylation (CYP1A1/2 and CYP3A1), 2β-hydroxylation (CYP1A1 and CYP3A1) and androst-4-ene-3,17-dione-associated monooxygenase (17-testosterone hydroxylase, CYP2B1 and CYP3A1) (Fig. 1).

In humans, correspondingly high levels of CYPs would predispose an individual to cancer risk from the widely bioactivated tobacco-smoke procarcinogens. Moreover, many of these could act synergistically with β-carotene as CYP inducers to impose a co-carcinogenic effect, particularly in genetically predisposed individuals who inherit the 'at risk' genotypes of xenobiotic metabolizing enzymes⁹. Indeed, studies have associated an increased risk of lung cancer with the induction of aryl hydrocarbon hydroxylase and/or polymorphisms in CYP1A1 (ref. 10). Although β-carotene is known to increase

the levels of phase II detoxifying enzymes such as glutathione *S*-transferase Mu and glutathione peroxidase enzymes, smokers with the CYP1A1 exon-7 valine polymorphism have significantly higher levels of PAH-DNA adduct; β-carotene intake does not significantly decrease these levels¹¹.

We used electron paramagnetic resonance to evaluate the precise contribution of CYPs induced by β-carotene on superoxide production. There was a significant association between the induction of CYP content in subcellular lung preparations and the overgeneration of superoxide yield (not shown), which could act synergistically with the peroxy radicals, nitrogen dioxide and hydroquinones that are contained in cigarette smoke. The pro-oxidant activity of β-carotene has also been unambiguously demonstrated at a high partial pressure of oxygen. Because this is highest in the outermost cells of the lung, these cells might be particularly subject to the pro-oxidant effect of β-carotene¹².

We found in a medium-term bioassay with BALB/c 3T3 cells that β-carotene enhances the conversion of the prototype benzo(*a*)pyrene to the ultimate carcinogens (our unpublished data). This co-carcinogenic activity of β-carotene is in line with the boosting effect of β-carotene itself on activating enzymes during cell growth.

Although cancer chemoprevention cannot rely merely on the control of antioxidants, there have been proposals to take advantage of the radical-trapping ability of β-carotene (and probably of other carotenoids) to try to decrease the incidence of lung cancer in humans. We postulate that the paradoxical effect of increased morbidity and mortality observed in the clinical chemoprevention trials is probably due to the co-carcinogenic properties of β-carotene and its ability to generate oxidative stress¹³. We think that our findings are relevant to public health policy and that they should be considered before widespread supplementation with these micronutrients is recommended.

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Growth of nanotubes for probe microscopy tips

Carbon nanotubes, which have intrinsically small diameters and high aspect ratios and which buckle reversibly, make potentially ideal structures for use as tips in scanning probe microscopies, such as atomic force microscopy (AFM)^{1–4}. However, the present method of mechanically attaching nanotube bundles for tip fabrication is time consuming and selects against the smallest nanotubes, limiting the quality of tips. We have developed a technique for growing individual carbon nanotube probe tips directly, with control over the orientation, by chemical vapour deposition (CVD) from the ends of silicon tips. Tips grown in this way may become widely used in high-resolution probe microscopy imaging.

Our approach to growing individual nanotube probes involves flattening a conventional silicon (Si) tip at its apex by contact AFM imaging and anodizing it in hydrogen fluoride⁵ to create nanopores of 50–100 nm diameter along the tip axis. Iron catalyst is electrodeposited into the pores from FeSO₄ solution⁶, and nanotubes are grown by CVD with ethylene and hydrogen at 750 °C. The orientated pore structure was chosen for the catalyst support in order to control the direction of growth⁷ and enable the reproducible production of nanotube tips for imaging.

CVD nanotube tips are formed reproducibly after a reaction lasting 10 min (Fig. 1). They are usually too long to be used as tips, and are shortened by an *in situ* AFM technique^{1,2}. A typical field-emission scanning electron microscopy (FE-SEM) image of a nanotube tip after it was shortened and used for AFM imaging (Fig. 1a) shows a well-defined tube 480 nm long protruding from the Si tip apex. Nanotube tips produced under these conditions and viewed by FE-SEM have an average diameter of 10 ± 5 nm. Further characterization of these tips by transmission electron microscopy show that they are multi-walled nanotubes (MWNTs) with well-ordered graphene walls (Fig. 1b).

AFM measurements of the cantilever

oscillation amplitude versus the position above a substrate (Fig. 1c) show that these individual nanotubes buckle elastically, as observed previously for mechanically attached, arc-grown MWNTs¹. Buckling curves can be repeated many times for a given tip, demonstrating that both the nanotube and its attachment to the Si tip are robust, and can be used to verify *in situ* that the nanotube tip is used for imaging.

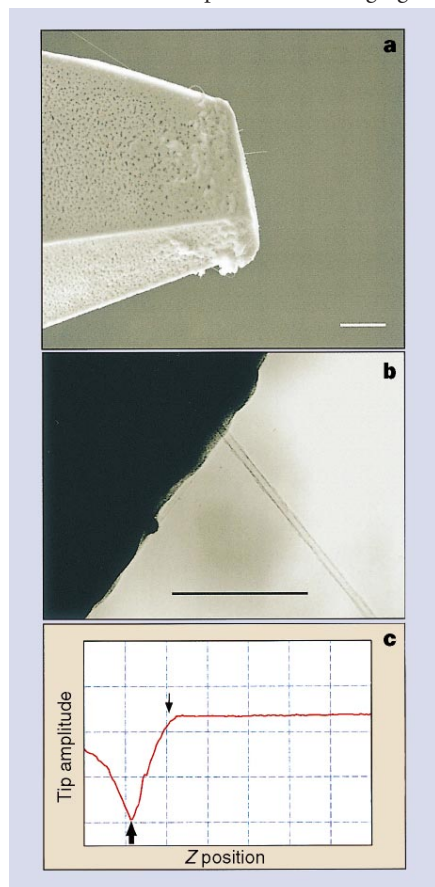


Figure 1 Characterization of CVD nanotube tips. **a**, FE-SEM image of a CVD nanotube tip that has been shortened for imaging. Nanotubes were grown from a uniform pore structure consisting of pores about 60 nm in diameter, with 150 pores per μm^2 . Scale bar, 1 μm . **b**, Transmission electron microscope (TEM) image of a CVD nanotube tip. The entire AFM cantilever/tip assembly with nanotube tip was mounted on a custom TEM holder for imaging. Scale bar, 100 nm. **c**, Tip oscillation amplitude (grid marks 5 nm apart) as a function of height above the sample (Z position; grid marks 2 nm apart) recorded in force calibration mode with a Nanoscope III (Digital Instruments). The right of the plot corresponds to free oscillation of the tip above the surface. As the tip approaches and begins to tap the surface (thin arrow), the amplitude decreases to zero. The oscillation amplitude increases again after the nanotube buckles (thick arrow). CVD nanotubes were grown in a tube furnace. Tips (with catalyst) were heated by 15 $^{\circ}\text{C}$ per min to 750 $^{\circ}\text{C}$ in a flow of 950 STP cm^3 min^{-1} argon and 40 STP cm^3 min^{-1} hydrogen. At 750 $^{\circ}\text{C}$, 10 STP cm^3 min^{-1} ethylene was added for 10 min, and the furnace was cooled at 15 $^{\circ}\text{C}$ per min in 500 STP cm^3 min^{-1} argon.

The imaging performance of CVD nanotube probes has been characterized using gold nanoparticle standards⁸ and immunoglobulin M. Gold nanoparticles with mean diameters of 2 and 5 nm were imaged on mica, and the image widths were used with a two-sphere model to estimate the tip resolution. The results show that we can routinely obtain very high-resolution tips with end radii of 3–6 nm. This is an improvement on our previous MWNT tips², our single-walled nanotube⁴ (SWNT) bundle tips, and commercial Si and Si_3N_4 tips, whose radii are generally greater than 5–10 nm. Our tips are also very robust and do not readily contaminate (in contrast to the sharpest Si and Si_3N_4 ones). They can be used several times and, when a tip ultimately fails, the carbon is removed by oxidation (at 500 $^{\circ}\text{C}$ for 20 min) and a new tip is grown by CVD. Tips have been through this cycle 20 times with no loss of yield or resolution.

These single nanotube tips may also be used for imaging biological macromolecules. For example, intermittent contact AFM images of IgM (Fig. 2) show excellent resolution. Routine images show the pentameric structure, including five external pairs of antigen-binding fragments and five internal crystallizable fragment domains⁹. Images occasionally exhibit a loop structure connecting two of the five domains that might correspond to the joining loop⁹. Higher-resolution AFM images have been

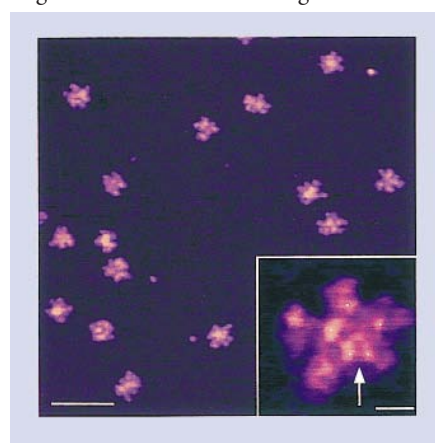


Figure 2 Imaging IgM macromolecules with a CVD nanotube tip at high resolution. Apparent structural differences in individual IgMs are due to absorption in different orientations. Molecular height ranges from 2.5 to 3.5 nm. Scale bar, 100 nm. Inset, high-resolution image of IgM. Small white dots, crystallizable Fc fragments; white arrow, possible position of the joining loop. Scale bar, 10 nm. Images were recorded in tapping mode in air with CVD nanotube tips grown on force-modulation-etched Si probes. Samples were prepared from mouse IgM (>95%, Sigma) by diluting to 2 mg ml^{-1} in 0.05 M phosphate-buffered saline solution, pH 7.0; 25 μl of the solution was dropped onto freshly cleaved mica, allowed to stand for 1 min, rinsed with water and dried with nitrogen.

reported using commercial tips^{10,11}, but only of densely packed arrays of proteins. When individual molecules have been imaged with these same tips, the resolution is lower¹¹.

Individual CVD nanotube tips routinely show higher-resolution images of isolated protein molecules than our previous results with MWNT and SWNT bundle tips (consistent with measurements on gold nanoclusters) and room-temperature AFM data with commercial tips¹², and are comparable to the high-resolution data obtained at low temperatures by cryo-AFM¹³. Our results reflect the small radii, cylindrical shape and low adhesion forces (typically less than 30 pN) of individual MWNT tips.

The high resolution of individual MWNT tips means that they could be used for imaging nanostructures and functional mapping when the ends are modified³. This CVD method also could be implemented on a large scale, making nanotube tips widely accessible. The resolution may be further improved by preparing smaller-diameter SWNT tips^{14,15}, leading to their use in many areas of biology and chemistry.

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An octopus-like obsession with loyalty

American Science in an Age of Anxiety: Scientists, Anticommunism, and the Cold War

by Jessica Wang

University of North Carolina Press: 1999.

375 pp. \$49.95, £37.50 (hbk), \$19.95,

£14.95 (pbk)

Daniel J. Kevles

In 1946, John and Hildred Blewett were invited to join the staff of the new Brookhaven Laboratory. Both were physicists who had worked on secret projects during the Second World War but, even though they would be engaged in unclassified research, their employment at Brookhaven required security clearance. The Federal Bureau of Investigation (FBI) questioned the Blewetts extensively about their politics and associations. David Lillienthal, a member of the Atomic Energy Commission (AEC), confided to his diary on hearing of the Blewetts' plight, "... the fact that ten years ago a scientist contributed to the defense of the Scottsboro boys, or believes in collective bargaining or the international control of atomic energy — such things as these are solemnly reported and regarded as 'derogatory information'."

The Blewetts ultimately got a hearing, and the AEC authorized them to work at Brookhaven on an uncleared basis. Even that limited disposition of their case had required the intervention of influential friends and taken seven months, and during that time they were reduced to living virtually on charity.

The Blewetts' story is only one of several harrowing encounters between scientists and the postwar national-security apparatus described by Jessica Wang, a historian at the University of California in Los Angeles, in her informative and eye-opening *American Science in an Age of Anxiety*. Some stories, like the Blewetts' ordeal, comprised *sub rosa* controversies fought in the shadowy interweavings of the FBI, academic and industrial contractors and AEC loyalty boards. Others involved bitter public confrontations between the House Un-American Activities Committee (HUAC) and politically left-leaning scientists, including, notably, the physicist Edward U. Condon, when he headed the National Bureau of Standards.

Covering mainly 1945–50, Wang's probing account of these episodes surpasses most others, since she draws on numerous public and private sources, including FBI records she obtained through the Freedom of Information Act. Her book is a graphic and disturbing analysis of how scientists became caught up in the octopus-like obsession with loyalty and security that pervaded American



life in the early years of the Cold War.

Yet Wang marshals her hard-won evidence in service of a larger political story characteristic of the United States at the beginning of the Cold War: how the tentacles of the octopus squeezed much of the life out of left-leaning activism in the politics of postwar policy-making, in this case for science.

Wang reminds us that, immediately after the Second World War, postwar science policy, especially in the nuclear arena, was vigorously contested. Key members of the wartime scientific leadership, like the conservatively inclined Vannevar Bush, advanced proposals for an AEC that would have been dominated by the military and, like Bush's proposed National Science Foundation, insulated from direct democratic control. But a number of scientists, many of them on the liberal left, fought openly for a civilian-controlled AEC responsive to the democratic political system. More generally, they battled for international scientific cooperation in atomic energy and a science policy responsive to social needs. These initiatives gave rise to numerous local scientific political action groups that soon joined together in the Federation of American Scientists (FAS).

Wang demonstrates that the growing

absorption with keeping atomic secrets incited attacks against these groups from conservatives, and surveillance by the FBI. The bureau monitored FAS chapter members and obtained information on chapter activities using informants. Its agents in turn fingered suspect chapter staff to selected FAS officers, who removed some of them from the staff to discourage red-baiting of the organization.

The assaults of the HUAC were energized in part by its ranking Republican member, Congressman J. Parnell Thomas, who was convinced that a civilian-controlled AEC would be security-loose. Politically savvy scientists took the HUAC's condemnation of Condon, an outspoken advocate of scientific cooperation with the Soviets, to mean that Thomas intended to reclaim atomic energy for the military.

Wang, with supple understanding of the relationship of executive agencies to congressional committees, points out that the Joint Committee on Atomic Energy was eager to protect the AEC from Thomas's attacks. However, Senator Bourke Hickenlooper, an Iowa Republican and head of the joint committee, pressured the AEC to forestall Thomas by tightening its security procedures. As far as Hickenlooper was

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concerned, only those beyond all suspicion should be granted clearance, regardless of the rights of suspects. Lillienthal, beleaguered, flawed, but understanding the stakes better than most, wrote in his diary that he would be "God damned if I would start lynching these poor devils just because Hickenlooper or anyone else didn't have the backbone to insist on decency in these things".

The FAS intervened on behalf of individual scientists facing clearance difficulties. It also lobbied the AEC to establish fair rules and procedures for dealing with security cases, holding that in practice the agency's security operations were often subversive of individuals' rights. Wang notes that, while the procedures improved, suspect scientists remained disadvantaged by having to deal with hearsay evidence about, for example, their associations, relatives and reading matter. The American Association for the Advancement of Science, the National Academy of Sciences and the FAS each attempted to forge a position on the threat posed by loyalty and security procedures to civil liberties. But in the end, Wang shows, each did little more than advance tepid recommendations for procedural reforms.

Using the records of the National Academy of Sciences and other documentary sources, Wang gives a remarkable account of the academy's refusal to take a strong public stand in defending Condon against the defamatory methods of the HUAC. She also

illuminates the controversy that arose when, in 1949, the AEC wanted all its fellowship holders to sign a loyalty oath and a non-communist affidavit. The academy, whose National Research Council selected the fellows for the AEC, accepted the ruling; but when, in the summer, the Senate mandated that all recipients of AEC fellowships undergo FBI investigations, the academy drew the line, forcing the AEC to compromise.

According to Wang, the loyalty and security procedures established by the Truman administration helped prepare the way for McCarthyism in science. This included applying political criteria in decisions on research grants, requiring loyalty oaths for members of university faculties, and denying passports to suspect Americans such as Linus Pauling and visas to suspect foreigners such as Paul Dirac.

Wang seems at times to push her case too far. She claims, for example, that, together with its anticommunist justifications, Truman's loyalty programme even "validated the more extreme actions of HUAC and, later on, Senator McCarthy". Nevertheless, her overall thesis is convincing: with their increasing focus on loyalty procedures, groups ranging from the FAS to the National Academy of Sciences avoided public or even internal discussion of the 'real questions' of what constituted loyalty in a democratic state. They thus permitted clouds of disloyalty to hang over politically progressive dis-

senters and contributed to the discrediting not only of them but also of their ideas. □

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Beware the diseases of the will, my child

Advice for a Young Investigator

by Santiago Ramón y Cajal, translated by Neely Swanson and Larry W. Swanson
MIT Press: 1999. 176 pp. \$22.50, £15.50

Pere Puigdomènech

Science as a career has never been an easy choice for a young person. For centuries it was the domain of philosophers and the enlightened rich; it only became a job at the turn of this century. Now, when unemployment and underemployment have become commonplace for young postdocs, how is it possible to envisage science as a choice? It may seem surprising to seek the advice of someone who wrote about this question a century ago, but the author is Santiago Ramón y Cajal, in many respects a unique personality in science.

The son of a country doctor, Cajal (1852–1932) was born in a little village in the north of Spain. He trained as a doctor himself and saw military service as a medical officer in the Cuban War of Independence before becoming professor of histology at several Spanish universities and developing a deep interest in the study of nerve cells. A critical and passionate writer, Cajal was so precise in his work that his drawings are still used in present-day publications and he remains one of the most cited authors of the life sciences. He was awarded the Nobel prize for medicine in 1906, and to this day is the only Spanish scientist to receive the prize for work done in his own country. He was active in promoting science, serving for more than 20 years as president of the Junta de Ampliación de Estudios, one of the first European institutions designed to promote the education of young scientists by helping students to travel and carry out experiments.

Cajal's *Advice for a Young Investigator* includes chapters on the qualities needed to become a scientist, the problems the young investigator may encounter and the way to write scientific papers. The book's curious subtitle in Spanish — *The Tonics of Will* — is very typical of this author's ideas. The will is, according to Cajal, the main feature that the young scientist has to cultivate.

One hundred years after this book was written, it evokes mixed feelings.

On the one hand, most of the advice and comments on the practice of science are perfectly valid

Rainbow reptiles

All the colours of the rainbow are to be found among the world's reptiles and amphibians, judging by a stunning portfolio of pictures of 91 species by the Japanese photographer Ryu Uchiyama (*Reptiles and Amphibians*, Chronicle,

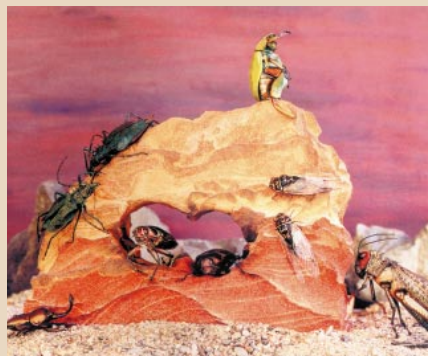
\$12.95, £8.99). The New Caledonian giant crested gecko, *Rhacodactylus ciliatus* (below), can vary in colour from brown and yellow to blue. It lives in trees and was thought to be extinct for more than 100 years, until it was rediscovered in 1994.





After Aesop

The Illusion of Orderly Progress (Knopf, \$20) is a collection of entomological compositions by the artist Barbara Norfleet. In the foreword, E. O. Wilson describes Norfleet's work as part of the tradition of animal fables which allows human nature to be scrutinized dispassionately. In the piece "My tribe is better than your tribe" (above), shining leaf chafer beetles (*Chrysina macropus*) confront metallic wood-boring beetles (*Euchroma gigantea gigantea*). In "The myth of coupling" (right), the two metallic wood-boring beetles (centre) are otherwise engaged while the single shining leaf chafer beetle straddles the stone alone. Norfleet is director and curator of the photography collection at the Carpenter Center for the Visual Arts at Harvard.



today. His description of the scientific method and the necessary attitude towards experiments and theories, for instance, are enriching for any present-day scientist. Anyone would recognize colleagues in his amusing descriptions of the different "diseases of the will" — sufferers include contemplators, bibliophiles, megalomaniacs and instrument addicts. His insistence that a young scholar should not be put off by the view that in science "the most important problems are solved" is also interesting. After what has happened during the past century in biology, one wonders what Cajal would think about present-day discussions on the 'end' of science.

On the other hand, the book is sometimes deliciously anachronistic. It strongly recommends studying foreign languages, especially German, "because it must be admitted that Germany alone produces more new data than all other nations combined when it comes to biology". And he is completely politically incorrect when he recommends as the ideal wife for a scientist one who "belongs

to him, whose best dowry will be a sensitive compliance with his wishes, and a warm and full-hearted acceptance of her husband's view of life". This advice is out of place in our labs full of young women but, from a historical point of view, the whole chapter deserves consideration.

The same is true when he praises patriotism as a source of motivation for the young scholar. Maybe some of these aspects are lost in the translation that converts nineteenth-century Spanish into modern English, and by the deletion of the last chapters, containing his analysis of the reasons for Spain's lack of standing in world science. Many of his comments in these chapters are, unfortunately, perfectly valid today.

The book was written by a person who had to work very hard to achieve an international standing in science, and who came from a country that was struggling to get away from its decadent imperialist tradition. He succeeded in building an easy relationship with the international scientific com-

munity and, following a rigorous methodology, he became influential as few other scientists have been.

Bearing in mind the distance in time and culture, you are left with the feeling that a high proportion of his advice is valid. It is written in the candid style of a person devoted to science and willing to help young people on the verge of making a decision that was as difficult a century ago as it is today. □

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Also new in translation

Of Flies, Mice & Men: On the Revolution in Modern Biology, By One of the Scientists Who Helped Make It

François Jacob, translated by Giselle Weiss
Harvard University Press, \$24

"It is just wonderful to read about genetics and to be reminded of details from the classics one has almost forgotten. If there were more books like this, genetics might not be under such an attack as it is now. It would be part of European culture". Benno Müller-Hill, *Nature* 386, 668–669 (1997)

And some contemporary advice for graduate students

A Student's Guide to Graduate School in the Sciences

by Dale F. Bloom, Jonathan D. Karp & Nicholas Cohen

Oxford University Press, \$16.95, £11.99 (pbk)

German science admits to fraud

Der Sündenfall: Betrug und Fälschung in der deutschen Wissenschaft [The Fall of Man: Fraud and Falsification in German Science]

by Marco Finetti and Armin Himmelrath
Raabe: 1999, 261 pp. DM34

Alison Abbott

German science lost its innocence two years ago with the exposure of what is probably Europe's worst case of scientific fraud: the now infamous Friedhelm Herrmann and Marion Brach stand accused of brazen fabrication of data in scores of peer-reviewed publications over many years.

Thirty-something Brach has admitted guilt, but says she was taught to cheat by Herrmann, who had been her mentor, scientific collaborator and lover. The "web of sex, violence and intrigue" that bound her to Herrmann was the breeding ground for the deceit, she claims. Herrmann, 11 years her senior, says Brach had not told him that she was making up results.

The case seemed to release pressure in a fermenting barrel, for German newspapers

have since reported a stream of new scientific fraud cases. These include the scientifically important and much-reported case at the Max Planck Institute for Plant Breeding in Cologne, where a technician was able to deceive the scientific world for years by fiddling a key assay, and the curious affair at the University of Giessen where a young veterinary scientist, stripped of his PhD, has been charged with trying to kill his whistle-blower by spiking his tea with digitoxin.

But according to the authors of this fascinating, if somewhat fatalistic book, cheating in German science is not just about the present — it has a long history, and possibly a solid future ahead of it.

Their thesis, coherently argued, is that cheating is both widespread and intrinsic to science, riddled as it is with what they alarmingly refer to as “diseases of science” — the competition for research funds, the pressure to publish and the fight for recognition in Germany’s rigidly hierarchical academic society.

They argue, correctly, that the German scientific community had, at least until now, kept its collective mind closed to the possibility that scientific misconduct could exist. ‘Idealists’ believed that science was too intrinsically pure to allow for cheating; ‘rationalists’ argued that science must always expose fiction because experiments are destined to be repeated; and ‘nationalists’ claimed that scientific fraud could never happen in Germany, where scientists have not been exposed to the same pressure as their US colleagues.

The authors efficiently dispense with these arguments. Some German scientists worked on Mengele’s experiments in the Nazi era, so science is not intrinsically pure; experiments can lie dormant in the literature for years before they are repeated, if at all; science is global, so there is no such thing as a national scientific culture.

They applaud the way research organizations responded to the Herrmann and Brach affair by designing codes of good scientific practice and efficient mechanisms for handling fraud within the research institution where it occurs, and by limiting damage done to the research community at large. But they fear that the unwillingness of universities to adopt the new rules will allow fraud to continue in a new regime of complacency: “*Problem erkannt — Gefahr gebannt*” (problem recognized, danger eliminated).

This judges too quickly. It is true that universities and research institutes initially displayed innate hostility to guidance from above — guidance, moreover, which seemed to demand a public acknowledgement that they could, in principle, harbour cheats. But universities are already accepting that rules must be set, if only because this is now a condition for eligibility for most sources of public research funds.

Der Sündenfall’s message may err on the side of alarmism, but it is certainly a good read, even though the science behind the scientific fraud is not always clearly described. It is expertly researched and its raw material has, by its very nature, a potent human element.

The book includes numerous case studies, beginning in the 1920s with Ernst Rupp, a physicist with the AEG company in Berlin, whose burning ambition to become a university academic, through fair means or foul, turned him into Germany’s first known perpetrator of scientific fraud. Rupp claimed that he had carried out an untested experiment designed by Albert Einstein in 1926 to investigate the properties of light.

Showing (apparently) the interference of electron beams, he (apparently) demonstrated the particle-wave dualism of light and matter. His claim precipitated scepticism among the academic community he sought to woo, since the technological hurdles to such an experiment were, at the time, immense. Over the next few years other German physicists were able to prove that he had lied. In his defence, Rupp produced a psychiatrist’s report saying that he suffered phases of “psychogenic trances combined with spiritual weakness”, during which “he unconsciously published reports about physical phenomena which had the character of fiction”.

It is interesting to note that in the good old days fraudsters, however bizarre their excuses, always admitted their guilt when overwhelmed by evidence. Their modern counterparts usually obey their lawyers’ advice to deny it to the bitter end. □

Alison Abbott is the senior European correspondent of Nature.

A further string to the believers’ bow

The Elegant Universe

by Brian Greene

Jonathan Cape: 1999. 428 pp. £18.99

John Maddox

A year ago, I fell into conversation with a young woman just embarked on a PhD stint at a British university. Her thesis adviser had assigned her a project in string theory, and I asked whether she believed that string theory would indeed answer all the questions of fundamental physics. “I don’t think so,” she said, “but the mathematics is interesting.”

Agnosticism such as this (and worse) is rife. For much of the past 15 years, almost the only rejoinder to scepticism has been the observation that Ed Witten, the Princeton theorist who has stepped into Einstein’s shoes at the Institute for Advanced Study, “is a believer”. But now the agnostics can read

Brian Greene’s remarkable book as well.

Greene is a regular physicist at Columbia, a practitioner of string theory of distinction and a proselytizer of the cause. (He is not to be confused with his near namesake, Michael Green, who with his colleague Julian Schwartz of Caltech caused a stir in 1984 by demonstrating that strings can reconcile quantum theory and relativity.) Greene’s contention is that the account given by string theory of the properties of the particles of matter is too good not to be true.

To be fair, Greene repeatedly acknowledges, although with decreasing frequency as the pages turn, that his high hopes for string theory may be disappointed. Perhaps he has shrewdly calculated that the sceptics will either have been won over by the repetition of the refrain “Strings are the cat’s whiskers!”, or that they will have fallen by the wayside before they reach the end — which is a long way from the beginning.

Greene starts with the frank declaration that quantum mechanics and general relativity are incompatible. That, in itself, is not a radical revelation: people have been trying to ‘quantize’ Einstein’s equations for a quarter of a century without success. Greene prefers to explain this failure qualitatively: Heisenberg’s uncertainty principle requires that quantum fluctuations increase without limit as the space accessible for the specification of physical variables shrinks indefinitely. That means that space itself, which is smooth on a macroscopic scale, is microscopically far from smooth — or “differentiable”, as mathematicians would say.

How does string theory resolve the difficulty? Elementary particles are no longer point-like objects, but tiny one-dimensional strings (which may be open with two loose ends or closed, like rubber bands) which, having tension, vibrate like piano strings. The energies of the normal modes of vibration then correspond to the masses of elementary particles (by the familiar rubric $E = mc^2$). They are all there. Electrons, quarks and the particles that transmit the various forces — photons, the heavy bosons of the electroweak theory and the gluons that mediate the strong nuclear force. And then, magic upon magic, there are also gravitons — the massless particles of spin 2 that are supposed to be the quantum particles of the gravitational field. That is how string theory unites gravitation with the other forces.

This picture, for outsiders, is also the stumbling block to understanding. A real string could not yield the riches of the known elementary particles. External disturbance of a vibrating string, perhaps by collision with another, would change a pure vibrational state into a mixture of all others, but photons do not turn into gravitons or into quarks of different kinds. Why do events of that kind never happen? Because the strings of particle theory vibrate in 10

dimensions — the four dimensions of relativistic space-time and six others of which we are unaware. Thus there is room for ample orthogonality to generate selection rules that prevent bizarre happenings between particles.

The most persuasive part of Greene's excellent book is that in which he persuades the reader that the problem of the six hidden dimensions is not a problem but a matter of perspective. A garden hose seen from a great distance looks like a one-dimensional object, but close up it is plainly a two-dimensional surface on which motion perpendicular to the length is perforce circular and repetitive. The length of the hose is Greene's analogy for an ordinary extended dimension; the perpendicular circular tracks stand for one of the six wrapped-up dimensions. If the radius of the circle (or of the garden hose) is small enough, it is recognizable only in close-up (which means at the highest energy).

How small are the compact radii? It seems to be agreed that the compact dimensions must be curled up with a radius in the neighbourhood of the Planck length, which is the constant with the dimensions of length formed from Planck's constant, Newton's gravitational constant and the velocity of light. Formally, the length is $\sqrt{(hG/c^3)}$, where h and G are the quantum and gravitational constants and c is the velocity of light. Numerically, the length works out at 10^{-35} m, which is small enough for Greene's purposes.

This is the kindergarten stuff of string theory, but Greene shrinks from no obstacle in the path of understanding, instead turning each into an opportunity to make the field exciting. The doctrine of supersymmetry has become an intrinsic part of string theory — otherwise 'superstring' theory. The implication is that there are as many fundamental particles yet to be discovered as are now known, but all of them are much more massive. String theory will fall if it is ever shown that they do not exist.

The essential step forward, and the cause of Greene's rekindled enthusiasm, was taken by Witten in 1995. He showed that the five alternative string theories then defined are essentially equivalent. A weak coupling constant (or the strings' equivalent of electric charge) in one will be the equivalent of another theory with a strong coupling constant. So, says Greene, the road points ahead to the age-old dream of predicting both the contents of the Universe and the properties of those contents. Already there are people working on the notion that black holes are merely very massive elementary particles.

Even if the dream proves false, Greene has brought an absorbing field of enquiry to vivid life. The supposition that particles are not points but strings fits well with familiar phenomena. The fact that all particles of matter have intrinsic spin (which may be

zero) has always provoked the question of whether spin is a property of particles or of space. The materialization of particles from apparently empty space is similarly provoking. String theory neatly answers them all.

So what lies ahead? Not even Greene is sure. String theory may not turn out to be the cat's whiskers he hopes. There are alternatives, such as Roger Penrose's twistor theory (which Greene reckons may say the same as strings). The most imaginative suggestion in this imaginative book is that the time has come to solve problems of quantum gravity in strictly quantum language and not by posing them in classical terms and then 'quantizing' them. Meanwhile, there is a whole raft of algebraic geometry to be done. The thousand and more people working in the field will need courage to do these largely thankless chores, but this splendid book will cheer them on their way. □

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The self-centred meme

The Meme Machine

by Susan Blackmore
Oxford University Press: 1999. 258 pp.
£18.99, \$25

Jerry A. Coyne

The meme, defined as "any unit of cultural transmission or any unit of *imitation*", was introduced by Richard Dawkins in the final chapter of *The Selfish Gene*. Dawkins' message was that any entity with the gene-like

properties of replication, variation and competition is a "selfish replicator" that can spread through populations by an analogue of natural selection. Memes qualify as replicators because bits of culture can be copied by imitation and compete with other units for human attention. Thus, despite the differences between memes and genes (genes, for example, are almost never passed to unrelated individuals), those memes most easily replicated and mimicked could proliferate, causing swift and important cultural change. Alarmed by the rampant and uncritical adoption of this analogy by philosophers, psychologists and the popular press, Dawkins downplayed the importance of memes in his later works.

However, in his foreword to Susan Blackmore's *The Meme Machine*, Dawkins notes that: "I was always open to the possibility that the meme might one day be developed into a proper hypothesis of the human mind, and I did not know how ambitious such a thesis might turn out to be." The thesis, called "memetics", has now arrived, and it is indeed ambitious. Unfortunately, Blackmore's book, aimed at both general readers and academics, proves to be a work not of science, but of extreme advocacy. Teeming with untestable speculations, indifferent to alternative theories and almost too grandiose to be taken seriously, *The Meme Machine* offers a convoluted — and wholly unsatisfying — explanation of cultural and biological evolution.

Blackmore defines memes as "units of imitation"; her examples from humans include songs, fax machines, books, alcohol, and any device, behaviour or idea that can be copied. Memes can also be combined into



DAVID NEWTON

"co-adapted meme complexes" that include a stimulus for imitation ("good girls don't have sex before marriage"), and into even larger "memeplexes", such as religion, with many copy-able elements. Like Sherlock Holmes, Blackmore sees our brain as a room of limited size that can store only so many memes. A meme's success depends not on its benefits to the carrier, but on its ability to be replicated, retained and imitated. As she declares persistently: "If a meme can get itself successfully copied it will." According to Blackmore, this shibboleth explains major aspects of our biology and culture, including oversized brains, language, technology, writing, contraception, adoption, altruism, vegetarianism, New Age ideas such as alien abduction, and consciousness.

Such an ambitious theory demands close scrutiny, but space restricts me to exploring only a few of Blackmore's views. She suggests, for example, that enlargement of the human brain began when natural selection improved the ability to imitate, followed by selection for ever more neurons to store successful memes. Blackmore contends that such selection has occurred only in hominids and birds that learn songs. Subsequently, memes further expanded our brain through sexual selection: as better imitators, bigger-brained individuals are more attractive mates and leave more offspring.

One immediately smells trouble. First, there is no evidence that brain-size increase had anything to do with memes — there are as many explanations (including language, social grouping, hunting) as there are evolutionists, and no way to judge which theory is best. Indeed, the meme hypothesis seems among the *least* likely. As others have noted, the serious proliferation of memes began roughly 30,000 years ago when humans commenced their march to larger social groups, writing and complex culture. The human brain, however, stopped enlarging after reaching its present volume nearly 500,000 years ago. Why did brain-size evolution stop so long before the heaviest rain of memes?

Although Blackmore deems memetics a scientific idea, nearly all of her suggested tests are either impossible to perform or unable to rule out competing theories. For example, she claims support for the sexual selection of memes, declaring that "... being highly articulate makes you sexually attractive. The history of love poems and love songs suggests as much, as does the sexual behaviour of politicians, writers, and television stars." This may be true, but Blackmore has forgotten sports heroes, rock stars and supermodels, also highly attractive but hardly known for eloquence. The combined data better

support the sociobiological theory that humans (especially females) are attracted by power and its attendant resources.

Narrower hypotheses have additional difficulties. A frequent problem is that the supposed meme seems to *discourage* its own propagation. Such a case is Blackmore's view that celibacy in Catholic priests is a meme that spreads by forcing clergy to divert their sexual energy into serving their religion. But what memes spring from this suppressed sperm? If priests beseech their flocks to forever refrain from reproduction, it is news to me. Other religions with non-celibate clerics, such as Islam, seem to have no trouble attracting believers.

Finally, Blackmore sees consciousness as a "selfplex", an insidious band of memes that conspire to give their carrier a false sense of self. Consciousness permits personal conviction, said to be especially good for encouraging imitation (for example, "I love beer"). However, consciousness cannot be illusory: as the American philosopher John Searle has argued, *thinking* one is conscious is identical to *being* conscious. Moreover, Blackmore's scenario denies the possibility of consciousness to meme-less animals, and fails to explain the hardest problem of human consciousness: subjective sensation. How can memes account for the pain I feel when I pinch myself?

But this is mere quibbling, for Blackmore's enterprise has two fatal flaws. First, she has got the chain of causation backwards. The claim that memes created major features of humanity is equivalent to the claim that the main force driving the development of better computers has been the self-propagation of software. In reality, computers are usually designed for speed and capacity, which then permits the development of new software. Similarly, the self-replication of memes does not mould our biology and culture; rather, our biology and culture determine which memes are created and spread. What a world of human psychology is obscured by Blackmore's mantra, "If a meme can get itself successfully copied it will"! To me, memetics boils down to the following obvious theory: ideas tend to spread if they cater to our desires to have love, comfort, pleasure, power, sex, the attention and admiration of others, a meaningful life and a way to evade the awful fact of mortality.

This brings us to the biggest problem: memetics seems completely tautological, unable to explain why a meme spreads except by asserting, *post facto*, that it had qualities enabling it to spread. One might as well say that aspirin relieves pain because of its pain-relieving properties. The most interesting question — *why*

some memes spread and not others — is completely neglected. Why did Christianity take hold during the waning days of the Roman Empire? You won't find the answer, or any way to attain it, in memetics. (This, by the way, makes memetics utterly unlike biological evolution. The spread of genes through natural selection is not tautological because one can predict their fate through their known effects on replication and the reproduction of their carriers.)

In a final effort to propagate her ideas, Blackmore constructs an extremely clever coadapted meme complex: "Evolutionary theory faced enormous opposition because it provided a view of humans that many humans do not like. The same will probably be true of memetics." Well, call me Bishop Wilberforce, but in my view memetics is but a flashy new wrapping around a parcel of old and conventional ideas. □

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Shady secrets of the Enlightenment

Sex and the Gender Revolution, Volume 1: Heterosexuality and the Third Gender in Enlightenment London

by Randolph Trumbach
University of Chicago Press: 1998. 484 pp.
\$35, £27.95

W. F. Bynum

Sex and bodies have become fashionable historical topics. Indeed, there are those who believe that historians are a prurient lot, preferring the bedroom to the laboratory, hospital, battlefield, palace and other sites where activities traditionally deemed more worthy of historical analysis take place.

Prurient or not, historians and social scientists quite correctly insist that bedroom behaviour can be exceptionally difficult to analyse. One of Randolph Trumbach's adulterers stuffed a handkerchief in the keyhole of her bedroom door, so that her servants could not spy on her. Even his randy men, who went with prostitutes in parks, ale-houses or dark alleyways, were unlikely to leave detailed accounts of their escapades. This, of course, makes eighteenth-century diarists such as James Boswell and William Byrd so attractive to historians, even if their class and status make them unsuitable for generalization about male behaviour down the ranks.

For the most part, then, Trumbach's account of heterosexuality in eighteenth-century London relies on other kinds of evidence, including newspapers, diaries, correspondence and imaginative literature.





The Bishop of London's consistory court heard suits for divorce and defamation. Magistrates' courts, Old Bailey papers and quarter-session rolls contain information on prostitutes and sexual crimes of violence as well as divorce. The archives of the Lock and Foundling hospitals reveal the fruits of illicit sexual activity among the poor, and Poor Law records are concerned especially with illegitimacy.

This diverse range of sources allows Trumbach to cut across class (since the poor did not leave diaries) and gender (both sexes sued for divorce and were treated for venereal disease). It also permits him to follow over time a range of parameters relating to the sexual lives of Londoners. These include prostitution, rape, bastardy, masturbation, courtship, domestic violence and adultery.

Whenever possible, Trumbach has quantified his data. Thus, we can learn things we never knew before about the occupations of men arrested for whoring or seducing women who subsequently offered their babies to the Foundling Hospital; the ages of spinsters making bastardy declarations in Chelsea or St Martin-in-the-Fields; and the gender and marital status of keepers of bawdy houses. The statistics are leavened by his reconstructions of the sexual histories of dozens of ordinary women and men whose passions and lusts, joys and sorrows, adventures and miscalculations brought them into the jurisdiction of the institutions of authority.

Through the subtlety of his analyses and the elegance of his prose, Trumbach has reached new heights in the study of historical sexuality. He has an impressive mastery of his material, on which he has been working for more than two decades, and an intimate knowledge of the geography and social history of eighteenth-century London. He has intriguing things to say about the changing

role of romanticism and sentimentality in both marriage and extramarital sex, and about the harsh realities of being an unmarried woman two centuries ago, when a proposal of marriage was sometimes an outcome of rape.

The nature of the available records of necessity leads Trumbach to a fairly bleak picture of human sexual conduct. Tolstoy reminded us long ago that "All happy families resemble one another, but each unhappy family is unhappy in its own way". Trumbach might have reflected on the fact that happy families, and happy relationships, rarely leave traces in the kinds of legal and institutional records he has mined. This is important, because Trumbach's picture of brutal and often violent sexual encounters between men and women is fundamental to the book's central thesis, one of breathtaking originality and, maybe, audacity.

That thesis is this: until about 1700, in London but probably also throughout Europe, neither effeminate, exclusive homosexuality, nor obligate heterosexuality, existed. Rather, the dominant sexual pattern was the one that we know existed in ancient Greece, and which Michael Rocke has recently argued also obtained in Renaissance Florence — about half the male population had same-sex experience during adolescence. Within this framework, sexual behaviour was regulated by age, and middle-aged men would routinely have slept with adolescent boys. Christian teaching castigated homosexuality, of course, but until 1700 or so, Trumbach suggests, these two morality systems existed side by side, with the official one dictating public and legal utterances even while the other one governed private behaviour. The consequences were the relative unimportance of prostitution, as men found other outlets for their sexuality, and a much more tolerant, if

rarely articulated, attitude towards sexual expression of all kinds.

Trumbach is silent about what ushered in the new regime of obligate heterosexuality. But after 1700, under this new regime, he insists, males felt compelled to exercise exclusive heterosexuality. Sodomy, even with women, was criminalized, and masturbation was medicalized into the dangerous practice of onanism, against which sermons and medical tracts were produced in profusion. Any underground homosexual activity that still existed was continued by a minority of exclusively homosexual males — the "third gender" of Trumbach's subtitle. Exclusive female homosexuality did not emerge, he believes, until about 1770; until then, females were denied any but heterosexual experience. This regime, which emerged within a single generation, lasted until about 1960, when gay consciousness and activism made homosexuality more visible, and bisexuality became possible once more.

Trumbach's bold scheme must be largely speculative, of course, given the large historical silences surrounding most aspects of human sexuality. The present volume is the first of a promised brace and focuses almost exclusively on the male-female consequences of what he frequently calls the "new male heterosexuality". Implicit in his scheme is the assumption that the old regime was somehow gentler, that males with homosexual outlets are less likely to need prostitutes, that sexual crimes of violence were less frequent and venereal disease and bastardy were somehow less common.

Much of his eighteenth-century evidence is consistent with this scenario: members of anti-vice societies of the 1720s certainly looked back to a time when their reformist activities would not have been needed (though one wonders what they would have made of the sodomy that Trumbach postulates was so common in Puritan Britain). Male libertines such as the Earl of Rochester and other Restoration rakes of the 1670s were far more likely to practise sodomy than were the eighteenth-century libertines who followed them. At the same time, one wants more from Trumbach about sexual mores in the earlier century, as well as trends in his period.

The full evaluation of Trumbach's stunning thesis must await his second volume, on the 'new' male homosexuality. Whatever one thinks of his explanatory framework, however, there can be nothing but appreciation for the achievement of this fine monograph. Trumbach has given voice to hundreds of ordinary men and women of eighteenth-century London, whose passions, lusts and tragedies have been reconstructed here with sensitivity and humanity. □

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The outward urge

The Sun, the Genome and the Internet, Tools of Scientific Revolutions

by Freeman Dyson
Oxford University Press: 1999. 124 pp.
\$22, £15.99

Walter Gratzer

"There's been a lot of progress since I was young," declared Ogden Nash, "but it's going in the wrong direction." Freeman Dyson will have none of that. Now in his mid-seventies, his concern for the future of humankind remains as lively as ever, and his optimism as invigorating. He has never been one to wait until his feet are wet before thinking up ways to stop the ship sinking. He was sufficiently exercised about the Earth's long-term prospects to moot a scheme for shifting it into a more salubrious orbit, and he even devised a scheme for evading the Big Crunch, should this eventuate in 10^{31} years or so, by way of an escape into a parallel universe.

One of his preoccupations now is with the practice and economics of space travel, for, as he shows, the US space programme has been direly misconceived and has by its reckless profligacy strangled other worthy activities, such as terrestrial astronomy. The mistake was to devote NASA's huge resources to putting a handful of people into low orbits, and such extravagances as bringing back lumps of rock from Mars, when nature has already left us generous supplies of the same material in the form of meteorites, mostly still reposing in the Arctic ice. Had the effort instead gone into developing more efficient space vehicles, driven, for instance, by laser propulsion or ram jets, there might even now be a community of explorers living on the Moon, and finding ways of adapting to the rigours of the environment.

Dyson's guess, based on a typical interval between discovery of a new land (America in 1492) and its settlement by outsiders (the arrival of the *Mayflower*), is that colonization of space could begin in about 2085. A self-sustaining ecology will follow the development of genetically engineered 'warm-blooded' (that is to say, heat-generating) trees to give warmth and shelter, as well as fuel and other useful products, secreted from their roots.

The settlement of space will become inevitable, Dyson believes, if only because human cloning and gene manipulation are also inevitable — for then human evolution will be given a sharp nudge and the new species of *Homo* that will emerge will have no desire at all to share one modestly appointed planet. So they will search for friendly corners of the cosmos: a moon of Jupiter, perhaps, or (most likely, Dyson thinks) the



archipelago of watery comets that make up the Kuiper Belt, or even the more distant Oort Cloud. Communities will spring up with libraries, concert halls and other amenities of civilized existence. And, no doubt, the yellow arches of McDonald's will greet the spaceships when they touch down. "One planet," Dyson concludes firmly, "is not enough."

Well, as the sage said: "Don't never prophesy unless you know", and Dyson concedes in his introduction that "experts, when they try to forecast the future, are usually wrong". Dyson is assuredly an expert: he has put his stamp on an unparalleled range of topics in mathematics, physics and technology. Jeremy Bernstein, physicist and writer, describing a casual but decisive intervention by Dyson in an intractable mathematical problem with which he was grappling, remarked that he could not conceive of how it must feel "to think with that rapidity and clarity ... Does everybody else appear to be going in slow motion?" But this is not all, for Dyson has also established himself, by his earlier books and articles, as a startlingly original social thinker and a proselytizer for science of rare panache and style.

This collection of his most recent cogitations is based on a series of four lectures given at the New York Public Library. The title reflects the three areas of research that he believes may dominate the next century.

Chapter one leads off with a brief discourse on how science has evolved and where it is heading. Although a theoretician, Dyson holds that the enterprise owes more to the spirit of the craftsman (like his boilermaker grandfather, with whom he feels a close kinship) than the philosopher. He believes scientists should develop their own tools, rather than buy them from instrument manufacturers, as he berates biologists for doing.

His strictures may cause some gnashing of teeth among the HUGOnauts, for instance, when he instructs them that wet chemistry is a crude, inefficient and reprehensibly expensive way to sequence DNA: he says the opera-

tion should be done on single molecules, degraded sequentially from one end, the liberated nucleotides passing into a mass spectrometer to be identified at the rate of one per millisecond. The exercise would then be complete in weeks, not decades, and the value of the project would be vastly enhanced because it would continue, with nominal added cost, to produce more genomic sequences. The priority should have been to develop such instruments — desk-top sequencers for all — and not to launch an enormous, labour-intensive programme, hobbled by the political imperative of a fixed target date. Dyson draws the parallel with the similarly inefficient, politically driven Apollo programme, which came to an end because it was not economically sustainable.

Dyson's arguments are illuminating, even if his strictures are sometimes less than just. Among biologists he makes one exception to his generalization — Fred Sanger. But Sanger did not develop apparatus: his methods of sequencing were the very apotheosis of wet chemistry. The miracle (as it must seem to the younger generation) of the achievements that brought him two Nobel prizes was that they employed little more equipment than a supply of filter paper. Biologists (depending on how one defines them) did, however, extend the frontiers of X-ray crystallography. As for the X-ray microscope — another bench-top tool, which, Dyson suggests, should supplant that crystallography — some fairly strenuous efforts have indeed been (and are being) made to construct one that works to any effect. And if biologists had had to master the skills in physics and engineering to build their own 800-MHz NMR spectrometers, there might have been little time left over for solving structures — no trivial task either.

Dyson's concerns now are centred on what technology might do to bring about a more equitable society. He supports, for instance, a charitable organization called SELF (Solar Electric Light Fund), which is developing cheap solar storage cells to bring electric light to isolated villages. Technology, he argues, has been for us a liberating force, which has done away with the tyranny of domestic service. You might be persuaded that the developed world, at least, is peopled only by the haves and the have-mores, if you were unaware of the widening income gap and the emergence of a disaffected population earning its crust by servicing the demands of the rich. Dyson recognizes that, in the poor and overpopulated countries, technology has served only to distance the privileged minority even further from the undereducated masses; he ponders the question of how the Internet and the World-Wide Web, which are already creating a commercial jungle and erecting ever-higher social barriers, might be tamed and made to serve the ideals of social justice.

In his epilogue, Dyson ruminates on the ascendancy of machines in our lives. A watershed for him was the defeat of Gary Kasparov by the chess-playing computer, Deep Blue, in 1997, a profoundly dispiriting event for many of us. Inevitable, says Dyson, and, of course, viewed as art succumbing to brute megabytes, tragic. But even here Dyson sees the promise of something greater to come, of art combining with machine to produce a new and deeper beauty. Let us hope so.

So, as the Reverend Sydney Smith observed during a time of trial, "cheerfulness will keep breaking through". "The game of evolution", Dyson assures us in his peroration, "will in future be played by humans and machines playing together. The landscape of cyberspace offers us as much scope for artistic creation as the landscape of a chessboard."

You may not be entirely persuaded by these assurances, but you will certainly feel the better for a bracing hour or two in Freeman Dyson's company. □

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Turning evening into morning

Time of our Lives

by Tom Kirkwood

Weidenfeld & Nicolson: 1998. 287 pp. £20

John Bayley

It's a grand life if you don't weaken, as they used to say. Or if you don't get anything wrong with you. In the old days you almost invariably did. TB, cancer, hypertension, pneumonia above all — they could carry you off without much fuss or bother between the fifth and seventh decades of life. But now, says Tom Kirkwood, we ought to become more conscious of the fact that those once almost inevitable shorteners of our lives don't come round for us any more. We should draw a deep breath and make the most of what used to be the evening of our lives. Make it a new morning.

Kirkwood's hearty tone and vigorous mode of exposition are agreeable to non-scientists, even if they do not necessarily grasp all the implications of a scientific argument. Take the little matter of cell ageing, on which much of the general optimism of Kirkwood's thesis depends. He tells the story of the conceited though charismatic French surgeon Alexis Carrel, a Nobel prizewinner who demonstrated that cells outside the body could be kept alive indefinitely in a properly maintained culture, and continue to grow. This seemed good news until Paul Moorhead and Leonard Hayflick demonstrated in their turn that actual body cells appear to be subject to what became known

as the Hayflick limit, the point beyond which cell reduplication did not continue. Could this be because cell ageing and final extinction are programmed as part of the ageing process? In other words, even if we escape the more obvious maladies of mortality, will we die just because our cells give up?

To help lay readers grasp the point, Kirkwood takes the analogy of an airliner hurtling down the runway towards take-off. Should something be still at fault after the elaborate pre-flight checks, a red light will come on in the cockpit, the crew will be alert-

ed and the flight aborted. But a wholly clueless outsider might draw the conclusion that the red light was itself the cause of the problem. Cell ageing, that is to say, may not be programmed, but may be the result of random damage or accident over a cumulative period, which will finally have the result of the whole immensely complex system packing up.

I am not convinced that this argument is particularly good news for septuagenarians who may be hoping to enjoy the time of their lives. What may be good news scientifically is



Mind where you put your molecules

Although *Memory: From Mind to Molecules* by Larry Squire and Eric Kandel is 69th in the Scientific American Library series (\$34.95, £23.95), it is highly individual. The book is a deftly crafted tour of the study of memory from a brief history of the science, via synapses and neurophysiology, to psychology and what the authors refer to as "the biological basis of individuality". And all this in ten short chapters.

As ever, Kandel and Squire write superbly even for the vaguely interested non-specialist. To crown it all, each chapter is fronted by a modern painting, the relevance of which is valiantly argued by the caption. Alberto Giacometti worked in cycles, creating from memory and then destroying successive works to produce a final version, such as "The artist's mother" (1950) shown here and fronting chapter 10.

perhaps best forgotten by those who are toiling on from day to day, and getting by as best they can. Causation, or its opposite, is itself an unsettling matter for the unscientific mind. Which would you rather feel: that your cancer has been genetically programmed, induced by stress or caused by viruses? Brought about by your own folly in smoking cigarettes, or — as Kirkwood himself appears to take for granted — brought about casually and spontaneously through the operation of chance? If it is put before them in this way, most people would rather not think about the matter at all.

Kirkwood — who is professor of gerontology at Manchester University — takes a different view, maintaining that the more we know about the ageing process the more we can “exercise a greater degree of personal control”. Common sense certainly tells us oldies to take it easy, to cut down a bit on food and alcohol and strenuous exercise, but we know this from the feel of our bodies rather than from what we read about the progress of science.

To declare an interest of my own, my wife, Iris Murdoch, died recently. For four years she had been suffering from Alzheimer’s disease, a brain disorder. Research was trying to find out why people died apparently not of, but with, this disease: what were the factors involved that produced a life-threatening situation? Apart from her mental troubles my wife had seemed to be in good health, eating and functioning normally. In a sense she seemed young rather than old, and I fed her, washed and cared for her as one would a three-year-old child. She seemed herself to attempt to adapt to this situation, to become good at it, just as she herself had been a good

person, as well as a great novelist and writer.

But quite suddenly, and as if more brain cells had switched off their circuitry, she stopped eating and drinking. My impression was that she wanted to be good at this too, as if swiftly to produce its best result. She died peacefully and quietly in my arms, and the medical certificate gave the cause as broncho-pneumonia. The doctor told me, between ourselves, that there was no sign of this, but one could hardly write down “starvation and dehydration”.

The function of consciousness in this ending remained mysterious, at least to me. One could hardly feel that Iris had retained any capacity “to exercise a degree of personal control”. But her body seemed to know what it wanted, and how to set about getting it. No doubt an illusion of the outsider, like the red light in the cockpit being the cause of the trouble, but it was strangely comforting to feel that in this most helpless and hopeless of disorders the body was not just dependent for its decisions on cellular malfunction or termination.

One of the charms of Kirkwood’s book is the vitality and insight conferred by its humour (humour that also survived in a 79-year-old woman transformed back into a three-year-old child). The best thing about growing old might be to have a good chuckle at it. As W. C. Fields, a lifelong hater of temperance-obsessed Philadelphia, did when he decided to make them write on his gravestone: “On the whole I’d rather be in Philadelphia”. That is one of the jokes Kirkwood tells in the course of an immensely shrewd but also light-hearted survey of our current prospects and situation, agewise:

and, as he more controversially maintains, what we ourselves ought to know and to do about it.

I feel sure he would agree with what might be termed the law of conservation of trouble. If we do live longer, remain more healthy and more alert, the young, for one thing, are not going to like it at all. They would much rather look after us than have to compete with us. Kirkwood, who evidently knows Africa well, has some pertinent concluding remarks on the current grave difficulties in tribal societies where the authority of the old, once questioned, is today not so much challenged as ignored. To the young there who want to live and work in go-ahead towns, the old have become irrelevant. It would be an irony if we could all start to expect to live to 100, and yet have to be sensibly programmed in some future social and scientific Utopia to die at 60.

One of Kirkwood’s engaging habits in this book is to preface his chapters with some suitable quotation from wise old poetry. (However young poets may die, poetry always knows how to grow old gracefully.) He omits, however, the ending of the Swinburne lyric, which thanks “whatever gods may be”,

*That no man lives for ever
That dead men rise up never
That even the weariest river
Winds somewhere safe to sea.*

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Sans hair, sans teeth...

A Means to an End: The Biological Basis of Aging and Death

by William R. Clark

Oxford University Press, £18.99, \$27.50

For those who want more of the same medicine, this immunologist and experienced popular-science writer’s latest book is on ageing. And he covers mostly the same scientific ground as Kirkwood in a similar way.

Molecular Biology of Aging

edited by Vilhelm A. Bohr, Brian F. C. Clark & Tinna Stevnsner

Munksgaard, Dkr400

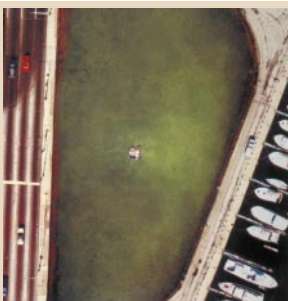
For the specialist, the edited papers presented at the 44th Alfred Benzon symposium held in Copenhagen in June 1998.

How and Why We Age

by Leonard Hayflick

Ballantine, \$14, £8.99

“Many a scientist has found immortality but none by Woody Allen’s preferred method — not dying. Avogadro lives on through his number, Planck his constant, Ohm his law. Leonard Hayflick, happily very much alive today, will be known to future generations for discovering the Hayflick limit, a phenomenon that seems partly responsible for the certainty that Woody Allen’s wish is one day going to be thwarted... Written



Hold infinity in the palm of your hand

A change in magnification of 10^{38} will take you from beyond the Galaxy into the nucleus of a carbon atom — a zoom that any film maker would envy.

Powers of Ten (Freeman, \$9.95) makes this journey in a ‘flipbook’ based on a short film by Charles and Ray Eames, better known as the designers of some of the century’s most famous furniture. These pictures on the human scale



make up the film’s pivotal central sequence, as we swoop down on, and into, the hand of an unsuspecting Chicago picnicker, rendered unconscious by his heavy lunch and even heavier choice of scientific reading matter.

in clear, nontechnical language, [Hayflick's book] is an excellent introduction to the scientific and demographic literature on this multifaceted subject." Tom Kirkwood, *Nature* 373, 484–485 (1995).

Lord of the flies

Time, Love, and Memory: A Great Biologist and His Quest for the Origins of Behavior

by Jonathan Weiner

Alfred A. Knopf: 1999. 290 pp. \$27

Yadin Dudai

William of Occam preached that "entities should not be multiplied beyond necessity", but did not forget to add: "there are many things that God does with more that he could do with fewer." The interplay between bold and cautious Occamism is the secret of successful science. This book is about a scientist who for the past six decades has navigated his way in a marvellously idiosyncratic style on the edge of Occam's razor, and swept biology, once and again, into new territories.

In the 1950s, in his adventures into the genetics of a virus, Seymour Benzer tore a window to the microcosm of the gene. In the late 1960s, toying with fruitflies in test-tubes, he altered neurobiology. Hundreds of hectic investigators at this very moment run knock-out mice in watermazes, slice the brains of transgenics or compose a paper to convince the world that their

mutant is the only clue to dementia. Many of them may not even have the faintest idea how it all started. Such is the fate of success: E. O. Wilson, cited by Jonathan Weiner, commented: "progress in a scientific discipline can be measured by how quickly its founders are forgotten." *Time, Love, and Memory* may hence be prescribed as an anti-amnesic potion to generations of neuroscientists.

Weiner's book is about Benzer and his quest for the origins of behaviour; it is also about a fascinating chapter in the history of science that, because the investigator and his organism played it low-key, was protected from attention for far too long — including probably the attention of the jury in Stockholm.

Benzer's lab at its most intensive period was off-off-Broadway, but it is there that a dominant scientific culture was founded. *Time, Love, and Memory* navigates us through Benzer's career with great detail, encompassing both scientific and personal life. We learn about the early days in physics (Benzer was among the discoverers of transistor technology); about the fascination with Erwin Schrödinger's *What is Life?*; and about the influence of another physicist, Max Delbrück, who started modern molecular biology by focusing on the tiny viruses — bacteriophage — as atoms of inheritance.

Benzer was drafted into the reductionist revolution. After years of almost manic research, he was able to present the world

with the first fine physical map of a gene. This turned him into the legendary 'atom-breaker of biology'. Other scientists would have probably capitalized on the success past retirement well into afterlife. Not Benzer. A loner by nature (a mutant?), Benzer consistently shies away from the crowds, especially when they attempt to join him. He is a scout, not a general, an obsessive wanderer into uncharted terrain.

Indeed, for a while he drifted into the more tranquil waters of RNA research. Delbrück, who started getting reprints, was quick to react: "Please ask Seymour," he wrote to Benzer's first wife, Dotty, "to stop writing so many papers... If he must continue, tell him to do what Ernst Mayer asked his mother to do in her long daily letters, namely, underline what is important." Benzer learned a lesson that should be included in the curriculum of every biology programme: 'beware of falling into the biochemical drain'.

He traded phage for fruitflies. The rest is history. In 1967 he published his first neuro-genetic paper: "Behavioural mutants of *Drosophila* isolated by countercurrent distribution". It is the epitome of an ingeniously simple approach to an extremely complex problem. The basic idea behind it all is: treat flies as atoms of behaviour. Induce single gene mutations. Devise a simple behavioural assay. Isolate mutants that flunk in the test. You now have an entry point into the dissection of behaviour.

Over the years, hundreds of mutants were identified. Weiner concentrates on three classes: *clock* mutants, which disrupt biological rhythms; *love* mutants, which disrupt courtship; and *memory* mutants. Nowadays, mainstream neuroscience mutates mice. Because scientists hate to learn from experience, in its infancy mouse neurogenetics fell into some of the pitfalls that *Drosophila* neurogenetics escaped from long ago, such as artefacts caused by genetic background, pleiotropism and hidden developmental lesions.

Weiner's book is well written and fun to read, although the question arises, who is the audience? This is scientific reportage and as such should not be expected to provide in-depth analysis of tenets and conclusions. Therefore, those who wish to find a critical assessment of neurogenetics should look elsewhere.

Many questions do deserve serious consideration. For example, what is the price of over-simplification? And what do genes tell us about behaviour? Consider, for example, the remarkable *memory* mutants. Those isolated so far affect the nuts-and-bolts of cellular plasticity. They are therefore 'plasticity mutants'. Memories are experience-dependent internal representations whose analysis must involve circuit and system levels. The expectation that neurogenetics *per se* would explain 'memory' thus neglects levels of

Dance at sea

Many seahorses form faithful long-term pair bonds which are reinforced by greeting rituals. The male and female may swim towards each other and dance together before changing colour and mating. The female plants sticky strings of eggs in the male's pouch, and he broods the embryos for up to six weeks before going into labour to produce fully independent young. It is no wonder that these bizarre creatures have fascinated people since at least the time of the ancient Greeks. The first guidebook to the world's seahorses does justice to their beauty and extraordinary biology, while being a user-friendly identification handbook and offering practical advice on the urgent need for conservation. *Seahorses: An Identification Guide to the World's Species and their Conservation* is by Sara A. Lourie, Amanda C. J. Vincent and Heather J. Hall (publisher, Project Seahorse, £19.95, \$32.95; distributed by NHBS; e-mail: nhbs@nhbs.co.uk).



complexity. With time, Benzer himself realized that *Drosophila* are not behavioural atoms but rather intricate individuals. He abandoned behavioural for developmental analysis.

No doubt, Weiner was captivated by the eccentric style of Benzerism, and this encouraged him to recite scores of amusing anecdotes. Whether all appeal to the general public, or only to a small cult, is a different question.

Another caveat is also appropriate: students who read the book should not expect mainstream science to follow Benzer's model. Most laboratory work is much more boring than the hunt for bizarre mutants in the forefront of science. Most mentors are much less imaginative, daring and permissive than Benzer. And, unfortunately, the days are long gone when an enthusiastic investigator shared plans without fear, and mutants without a lawyer. This was before business killed fair play.

As for Benzer himself — at the age of 77 he has just discovered a new mutant, *Methuselah*, that refuses to age. □

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Hitting the target

The Elusive Magic Bullet

by John Mann

Oxford University Press: 1999. 202 pp.

£18.99, \$29.95

Stephen Neidle

The use of medicines to combat disease almost certainly predates recorded human history. One can imagine our prehistoric ancestors, during the course of foraging for food, finding that whereas some plants and fruits were harmful, others could have beneficial effects. The Chinese developed particular expertise with herbal remedies, a tradition that is at least 5,000 years old and still thrives today.

The science of drug discovery has its origins in this tradition. Many subsequent cultures also contributed, with primitive pharmacy arising from the ancient Greeks, and notably from the Arabic world. The Dark Ages onwards saw these elements intertwining with the pseudo-science of alchemy, a combination that dominated materia medica until the onset of modern science at the end of the eighteenth century. All this and more was the subject of John Mann's earlier book, *Murder, Magic and Medicine*, which focused on the use of natural products through the ages, for both base and noble purposes. Mann, a distinguished natural product chemist himself, succeeded in the difficult task of writing an entertaining yet



Tracing the development of lines

The artistic development of talented and less talented children was compared over a ten-year period by the developmental psychologist Constance Milbrath. She assessed the ages of development of different elements of drawing, including form, spatial relationships and composition. The study has been written up in a technical book, *Patterns of Artistic*

Development in Children: Comparative Studies of Talent (Cambridge University Press, £40, \$59.95). Milbrath describes the development of artistic talent as being dependent on both figurative and cognitive abilities. Here, a talented 11-year-old has drawn the back views of two people and a dog.

erudite book, with appeal to readers across a wide spectrum of backgrounds.

His new book continues and expands on this theme and approach. Its focus is the discovery of twentieth-century medicines for the treatment of bacterial and viral diseases and cancer, and their historical context. Its style is a beguiling blend of science and historical detail, liberally spiced with anecdotes and accounts of the wider impact of these discoveries.

The "magic bullet" concept originated with Paul Ehrlich. Mann pays fitting tribute to Ehrlich as the true father of modern approaches to drug discovery, since he also effectively devised the concepts of therapeutic index, chemoreceptors and combination therapy. But it is the idea that there exist perfectly specific drugs to combat human disease that grabs the public imagination. One real strength of this book is Mann's critical examination of this idea, especially in the context of anti-cancer therapies, where public demand for magic bullets is a potent force.

The survey of biological approaches to cancer therapy succinctly covers such topics as interleukins, Ras farnesyltransferase inhibitors, angiogenesis and antibody-directed enzyme prodrug therapy. As Mann points out, this last approach probably comes closest to Ehrlich's original concept, even though it still awaits full verification in the clinic.

It is difficult to envisage a world without

even the simplest antibacterial agents to ensure that we don't succumb to a brief encounter with pathogens in our everyday life. Average life expectancy was only about 45 years at the turn of this century, when Alexander Fleming started as a medical student. One child in six did not survive beyond five years. Mann has provided a vivid account of the devastating impact that bacterial diseases had on the populace, right up to the Second World War, and the dramatic effects of the widespread availability of antibiotics such as penicillin and streptomycin. Some topics, such as the discovery of penicillin and its subsequent development into a clinically available drug, are rightly given extensive coverage. The problems of drug resistance and superbugs are also discussed in brief, but adequate, illustrated descriptions of antibiotics' modes of action and the molecular basis of resistance.

Surprisingly, in a book written by a chemist, there is not a single chemical structure to be found, even though there are numerous descriptions of them and their significant features. Perhaps the publishers felt that the inclusion of chemical formulae would intimidate potential purchasers.

Following the Second World War, the search for further chemotherapeutic agents with selective activity against particular organisms became the mainstay of much of the pharmaceutical industry. Many companies were effectively vast palaces of organic

chemistry and microbiology, to which other biological sciences were mere appendages providing the necessary *in vitro* and *in vivo* screens. Studies of the products of mould and microorganism metabolism and their chemistry dominated drug research. This approach resulted in the discovery of many effective antibacterial and some useful anti-tumour agents. Others often resulted from chance findings, notably the anticancer drug cisplatin.

However, by the late 1980s drug discovery was in a rut, with the realization that random screening was an expensive and ultimately unscientific way to find agents with significantly superior activity and selectivity. The subsequent revolution in our understanding of the molecular basis of many diseases has prompted fundamental changes in approach. The current, more rational approach is based on an understanding of pathogen and malignant cell and molecular biology. The impact of the human genome project on the treatment of human disease promises to be even more profound.

Mann's parting message is that all this takes us full circle, back to the essence of Ehrlich's approach, based on the idea of specific chemoreceptors.

Inevitably, a small book with such an ambitious agenda has to emphasize some topics more than others. I would have expected to see a fuller (and illustrated) account of the impact of structure-based drug design, especially since there are several excellent recent examples now in the clinic, such as the anti-HIV protease inhibitors. There is no real mention of combinatorial chemistry, now having a profound impact in industry and even academia.

However, these are minor caveats for an engrossing book which serves its intended audience well. It has much of value for the interested layperson, as well as students and even professionals wishing to have a gentle yet erudite introduction to the past, present and future of chemotherapy. □

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Drugs from other perspectives On the one hand:

Bitter Pills: Inside the Hazardous World of Legal Drugs

by Stephen Fried

Bantam Books, \$24.95

And on the other ...

In Quest of Tomorrow's Medicine: An Eminent Scientist Talks About the Pharmaceutical Industry, Biotechnology, and the Future of Drug Research [Die verspielte Zukunft]

by Jürgen Drews, translated from the German by David Kramer

Springer, \$29.95, £22.50

Taming the black dog

Malignant Sadness: The Anatomy of Depression

by Lewis Wolpert

Faber & Faber: 1999. 186 pp. £9.99

Charles B. Nemeroff

In the past decade, a number of books have documented personal experiences with depression or manic-depressive illness. There are the rare public figures willing and able to share their voyages to hell and back. There are the professionals (including mental-health workers) who have, after an episode of depression, dramatically changed their view of the disorder. And there are the articulate patients wishing to educate both the lay public and health-care professionals on the diagnosis and treatment of this devastating disease. There are probably more than 20 such volumes available, many of them excellent. Do we need another? In contemplating the request to review this book, I had to take into consideration my current tardiness in several projects. So why did I agree?

For the biologists, the answer is obvious, but to the rest of you, it is simply because the author is Lewis Wolpert. Wolpert is an accomplished developmental biologist, who has not only made remarkable research contributions, but has helped frame many questions in biological research, cutting across a number of disciplines. In view of his scientific prowess and his role as a philosopher-

scientist, it was simply too tantalizing for me to resist the temptation to preview what he writes about a disorder that I have spent most of my professional life studying.

I was not disappointed; there is much good here. As he himself now recognizes, one of the major problems with depression is the stigma attached to it by both the public and many health-care providers. His willingness to share the pain, self-doubt, hopelessness and helplessness that is pathognomonic of this disorder will help many non-sufferers of depression to understand, and many depressed patients to fight this "black dog", as Churchill termed it. Wolpert's description of the severe sleep disturbance, obsession with suicide, anxiety, inability to concentrate, diurnal mood variation and bewildering physical symptoms is compelling. His grasp of the literature is, not surprisingly considering his biological background, very topical and generally accurate. He conveys the public-health implications of depression in a very readable manner, which is no small task. He highlights several facets of depression that are generally not emphasized enough, including its remarkably high prevalence worldwide, and the morbidity and mortality associated with it. In the United States, suicide is the ninth leading cause of death, in spite of the experts' acknowledgement that many, if not most, suicides go unreported.

Wolpert's repeated emphasis on depression as a systemic disease that affects several other organs, including the heart, is a well-established finding that has not received the recognition it deserves. He is one of the first authors to repeatedly remind us that the



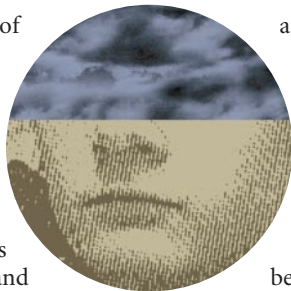
DAVID NEWTON

spouses and family of depressed patients also suffer terribly, and must be partners in the treatment. His cogent discussion of the classification of mood disorders and the current diagnostic process and measurements of disease, by categorical and dimensional measures, respectively, is a godsend. I found his discussion of the nature and seriousness of postnatal depression particularly valuable. He also does a very good job of summarizing the neurobiology of depression, with an emphasis on the role of alterations in serotonergic and noradrenergic neuronal systems, and of the hypothalamic-pituitary-adrenal axis in the pathophysiology of mood disorders.

What didn't I like? The author's understanding of the data from controlled clinical trials related to the treatment of depression was a bit disappointing. Perhaps I expected too much, but we get a relatively non-critical and superficial view of a very complex field. For example, Wolpert says that the literature reveals all antidepressants to be equally efficacious. Although this is technically true, in that no regulatory agency in any country has ever recognized one antidepressant as more effective than another, there is a raging controversy in the field over this issue. There are data that suggest that certain of the older tricyclic antidepressants, such as chlorimipramine, are more effective than some of the newer agents. This is obviously not a trivial point, and may relate to the older drugs' effects on more than one neurotransmitter system, compared with the newer, more selective agents.

Perhaps more important is the lack of communication of the following observation, which is well known among psychiatrists: most depressed patients treated with an antidepressant respond only partially or not at all. Unfortunately, many general practitioners and even psychiatrists do not use measures of depression severity to follow their patients' progress, and therefore never realize that their patients have, in fact, not completely recovered. It is also important to determine whether the depressed patient has delusional (psychotic) thinking; such patients do not respond to antidepressant monotherapy, and require combination treatment with an antidepressant and an antipsychotic medication.

Another minor disappointment is Wolpert's discussion of the role of psychotherapy in treating depression, and the (perhaps) related issue of placebo response of depressed patients. It is difficult for the uninitiated to understand how depression can, on the one hand, be as severe as it is described (severe functional impairment, increased risk of heart disease and stroke,



and increased risk of suicide) and, on the other, respond relatively well to placebo treatment. The answer lies, at least in part, in that the depressed patient assigned to the placebo group receives all of the treatment except the drug (the contact with a myriad of research personnel will be a form of therapy). In terms of the psychotherapies themselves, suffice it to say that the more severe the depression, the less likely that it will respond to psychotherapy alone. Moreover, as Wolpert points out, the decreases in concentration and cognitive dysfunction characteristic of severe depression hardly make the patient a candidate for psychotherapy. He aptly points out the important theoretical underpinnings of psychoanalysis and psychoanalytically oriented psychotherapy in the aetiology and treatment of depression, but surprisingly fails to highlight the lack of evidence for the efficacy of this approach.

I was not surprised by his attraction to genetics, as this field will probably shed considerable light on depression research over the next decade, perhaps more than any other

field. The completion of the Human Genome Project will provide the field with tools that will surely advance depression research. I believe that functional brain imaging will also contribute much, as will the burgeoning pipeline of promising therapies.

In summary, *Malignant Sadness* will stand up well with other personal descriptions of depression, including some he refers to in his volume: Kay Redfield-Jameson's *An Unquiet Mind* and Kathy Cronkite's *On The Edge of Darkness*. It is a fitting companion to others who have written eloquently on this subject, including Tracey Thompson's *The Beast: A Reckoning with Depression* and Martha Manning's *Undercurrents*. In view of the growing evidence for the role of adverse early experience in the pathogenesis of depression, the only solution may be to offer Wolpert a sabbatical to share his developmental biology expertise, together with his personal knowledge of this disorder, to help design experiments to uncover biological causes of this observation. □

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Starr book wins Los Angeles Times prize

Douglas Starr's book *Blood: An Epic History of Medicine and Commerce* was announced as the winner of the science and technology category of the 1998 Los Angeles Times Book Prize last Friday (23 April). Starr was one of five finalists in the science and technology category:

The Baltimore Case: A Trial of Politics, Science and Character

by Daniel J. Kevles
W. W. Norton, \$29.95, £21

"Daniel Kevles performs a signal service by applying his historian's acumen to the mountain of documents accumulated by all those investigations, and to his own detailed interviews with the participants, to give us an orderly narrative of the whole affair. He did so, he says, because it seemed to him likely to 'throw some light on science in late-twentieth-century American society.'" Jon Turney, *Nature* 395, 30-31 (1998).

Phantoms in the Brain: Probing the Mysteries of the Human Mind

by V. S. Ramachandran and Sandra Blakeslee
William Morrow, \$27, £17.99

"*Phantoms in the Brain* is structured around the life histories of patients with unusual, often bizarre, neurological disorders. The narrative structure of biography has undoubted pedagogic value, but the worst examples of the genre come uncomfortably close to modern-day equivalents of a travelling freak-show. To their credit, this pitfall is skilfully avoided by Ramachandran and Blakeslee, who keep the ideas they are seeking to

communicate centre stage." Raymond J. Dolan, *Nature* 396, 639-640 (1998).

Taking Wing: *Archaeopteryx* and the Evolution of Bird Flight

by Pat Shipman
Simon & Schuster, \$25.

"Shipman makes an outstanding analysis of the contribution of *Archaeopteryx* to discussions about the origin of flight." Jose Luis Sanz, Bernardino P. Perez-Moreno & Francisco J. Poyato-Ariza, *Nature* 393, 32-33 (1998).

Blood: An Epic History of Medicine and Commerce

by Douglas Starr
Knopf, \$27.50, £20

"While the value of a barrel of crude oil has fallen to \$12, an equivalent amount of blood is now worth around \$60,000. How this came about is lucidly recounted by Douglas Starr in this history of blood transfusion. The book is accessible to a wide-ranging audience." Fred S. Rosen, *Nature* 398, 303-304 (1999).

Mendel's Dwarf

by Simon Mawer
Harmony, \$23 (hbk); Anchor, £6.99 (pbk)

The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis

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Fas is a cell-surface receptor molecule that relays apoptotic (cell death) signals into cells. When Fas is activated by binding of its ligand, the proteolytic protein caspase-8 is recruited to a signalling complex known as DISC by binding to a Fas-associated adapter protein. A large new protein, FLASH, has now been identified by cloning of its complementary DNA. This protein contains a motif with oligomerizing activity whose sequence is similar to that of the *Caenorhabditis elegans* protein CED-4, and another domain (DRD domain) that interacts with a death-effector domain in caspase-8 or in the adapter protein. Stimulated Fas binds FLASH, so FLASH is probably a component of the DISC signalling complex. Transient expression of FLASH activates caspase-8, whereas overexpression of a truncated form of FLASH containing only one of its DRD or CED-4-like domains does not allow activation of caspase-8 and Fas-mediated apoptosis to occur. Overexpression of full-length FLASH blocks the anti-apoptotic effect of the adenovirus protein E1B19K. FLASH is therefore necessary for the activation of caspase-8 in Fas-mediated apoptosis.

Fas is a widely expressed cell-surface receptor molecule belonging to the tumour-necrosis factor (TNF) receptor family^{1,2}, which can induce apoptotic signals in Fas-expressing cells by binding with agonistic anti-Fas monoclonal antibody or with its natural ligand, FasL (CD95L)³⁻⁵. The mechanism of signal transduction of Fas-mediated apoptosis has been extensively investigated as a model of mammalian apoptotic cell death. Several proteins have been identified that bind the intracellular domain of Fas, designated the death domain, which is essential for signal transduction of Fas-mediated apoptosis². Of the Fas-binding molecules, the adapter protein FADD/MORT1, which has a death domain in its carboxy-terminal region, has an important function in Fas-mediated apoptosis⁶⁻⁹. FADD is recruited to Fas after stimulation of Fas¹⁰, and then binds caspase-8 (also known as FLICE/MACH/Mch5), a member of the mammalian caspase family through homophilic interaction between the amino-terminal domains of FADD and caspase-8, which are known as the death-effector domains (DED)^{11,12}. In the complex of Fas, FADD and caspase-8, which is called the death-inducing signalling complex (DISC)¹⁰, caspase-8 becomes proteolytically autoactivated by oligomerization^{13,14}, whereupon it stimulates other caspases, including caspases 3 (ref. 15), 6 (ref. 16) and 7 (ref. 17), by cleaving them. These downstream caspases cleave the death substrates¹⁸ that are central to apoptotic events such as morphological changes¹⁹ and DNA fragmentation²⁰.

Although it has been proposed that caspase-8 is proteolytically autoactivated in the DISC complex after oligomerization through its DED domain during Fas-mediated apoptosis^{13,14}, we suspected that some other molecule(s) apart from Fas and FADD could activate caspase-8 in DISC; this was based on the fact that the *Caenorhabditis elegans* protein CED-4 and its mammalian homologue Apaf-1 are required for the activation of *C. elegans* caspase CED-3 and mammalian caspase-9, respectively^{21,22}. It has been shown that the anti-apoptotic adenovirus protein E1B19K, which is unable to bind Fas, FADD or caspase-8, can indirectly prevent Fas-mediated and FADD-induced activation of caspase-8 (ref. 23), indicating that it may inhibit Fas-mediated apoptosis by binding an unknown protein(s) that is involved in the activation of caspase-8. In addition, Fas-induced DISC formation was barely detectable in

some Fas-sensitive cells expressing sufficient amounts of the known components of DISC²⁴, suggesting that there may be another component(s) in the DISC. Here we identify a new protein that binds to caspase-8, which we call FLASH (for 'FLICE-associated huge' protein). We show that FLASH, which contains a motif structurally related to CED-4/Apaf-1 and two tandem-repeated DED homologous domains, is required for the activation of caspase-8 during Fas-mediated apoptosis.

Identification of FLASH

To investigate the mechanism of activation of caspase-8 in Fas-mediated apoptosis, we screened a mouse T-cell complementary DNA library by using the yeast two-hybrid technique and two tandem-repeated DED domains of procaspase-8 as a probe. We obtained 76 β -galactosidase-positive clones from 2×10^7 library transformants. Classification of the positive clones by restriction analysis and nucleotide sequencing revealed that they contained thirteen independent clones of FADD, seven UBC9 clones, two c-FLIP/Casper/CASH short forms, and two other unknown clones, numbers 68 and 149. UBC9, which is one of the ubiquitin-conjugating enzymes (E2), binds Fas or other molecules in yeast two-hybrid experiments. A BLAST search suggested that clone 68 was a mouse homologue of *Gal4*. The other clone, clone 149, has no significant identity to any proteins in the GenBank Database but has a domain homologous to DED of caspase-8.

To obtain the full-length sequence of clone 149, rapid amplification of 5' cDNA ends by polymerase chain reaction (5'-RACE PCR) was performed using a cDNA library from WR19L12a cells, and then a cDNA library prepared from F9 cells was screened by colony hybridization with the fragments obtained by 5'-RACE PCR. Partially overlapping isolated inserts were ligated to give a cDNA containing a very long open reading frame (5,886 base pairs (bp)) with an initiator methionine (ACCATGG), in agreement with Kozak's consensus²⁵. We determined the initiation site by finding an in-frame stop codon 18 bp upstream of the putative initiator methionine in a G+C-rich 5' region of the cDNA. This gene encodes FLASH, a polypeptide of 1,962 amino-acid residues and of predicted relative molecular mass 219.1K (Fig. 1a).

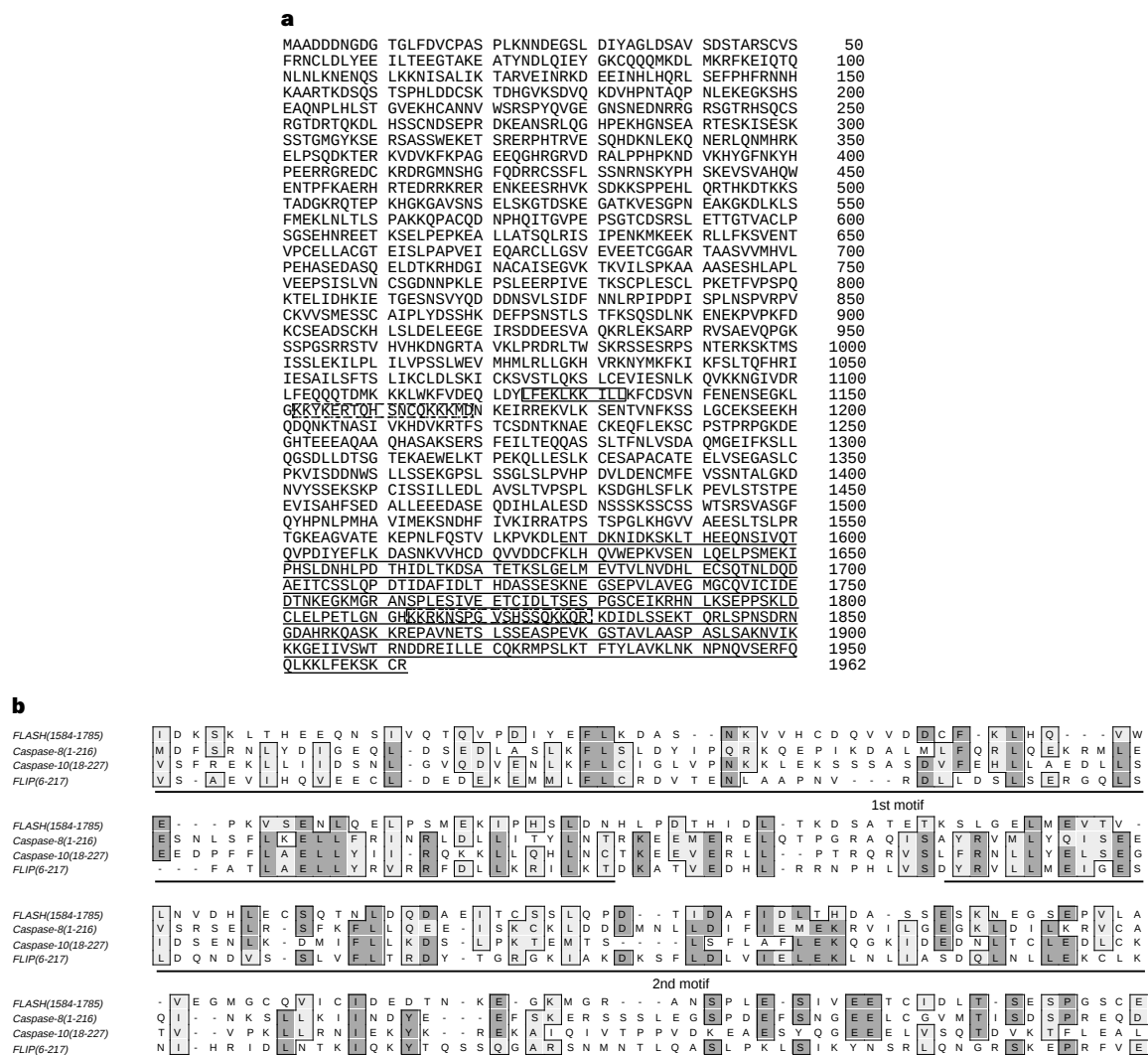


Figure 1 Structure of mouse FLASH and sequence comparison. **a**, The deduced amino-acid sequence of mouse FLASH. Numbers on the right indicate the amino-acid residue position. The original clone isolated by yeast two-hybrid screening is underlined. The box and dotted boxes indicate the putative nuclear exclusion

signal (NES) and the putative nuclear localization signal (NLS), respectively. **b**, Sequence alignment of amino-acid residues 1,584–1,785 of FLASH (DRD) with the two tandem-repeated DED of caspase-8, -10, and c-FLIP. Dark shaded boxes, identical residues; light shaded boxes, similar residues. The first and second

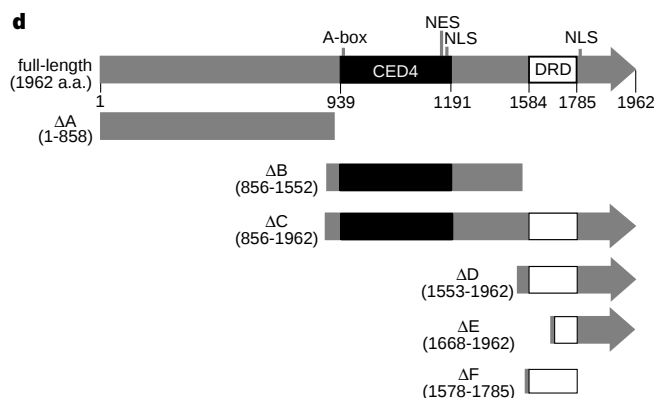
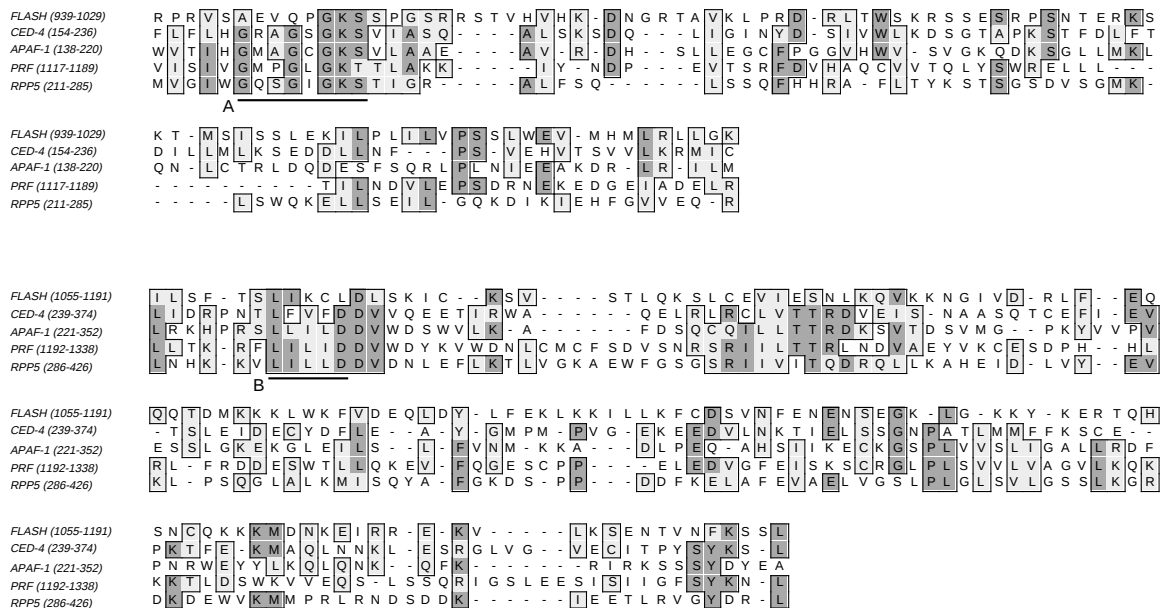
Sequence analysis of FLASH revealed that the C-terminal region originally obtained from a yeast expression library contained a domain significantly homologous to the duplicated DED of caspase-8 (sequence was 20% identical and 41% similar), although the similarity of the duplicated DED of caspase-8 to FLASH is less than that to c-FLIP and caspase-10 (Fig. 1b). Some consensus amino-acid residues in DED are not conserved in FLASH. We designated this DED-like domain of FLASH as DRD (for DED-recruiting domain), because FLASH interacts with the DED domain not only of caspase-8 but also of FADD through its DED-like domain (see later).

An ATP/GTP-binding motif homologous to the Walker's A-box consensus sequence was found in the central part of FLASH (amino acids 944–951; underlined in Fig. 1c)²⁶. The region at residue positions 939–1,191 containing the putative nucleotide-binding site showed weak structural similarity with CED-4, Apaf-1, tomato PRF and plant resistance-gene products (Fig. 1c). This region of FLASH, however, contains an extra stretch of 24 amino-acid residues at 1,030–1,054 as compared with other CED-4-homologous proteins, and appears to lack the Walker's B-box (Fig. 1c). The sequence identity and similarity between the region of FLASH before the insert (residues 939–1,029) and the corresponding

region of CED-4 were 18 and 38%, respectively. Those between the region of FLASH after the insert (amino acids 1,055–1,191) and the corresponding region of CED-4 were 15 and 33%, respectively. The amino-terminal segment of FLASH (residues 1–858; region ΔA in Fig. 1d) showed no significant homology to any protein in the GenBank Database.

We found an amino-acid sequence homologous to the nuclear-localization signal (NLS) in the C-terminal region of FLASH²⁷ and a nuclear-exclusion signal (NES)-like sequence²⁸ in the central region of FLASH (LFEKLKKILL) (Fig. 1a). The putative NES of FLASH may be functional because western blotting with rabbit anti-FLASH antibody indicates that most FLASH is in the cytoplasmic fraction (data not shown).

Northern blotting analysis revealed that FLASH was constitutively expressed as two transcripts (of ~4.7 kb and ~7 kb) in all adult mouse tissues examined (Fig. 2). Its expression was relatively high in the heart, brain, thymus, lung, testis (Fig. 2) and spleen (data not shown). Western blotting analysis with an affinity-purified rabbit anti-FLASH antibody detected specific bands at 220K in NIH3T3, Balb3T3 and FH2 cells and in mouse thymocytes (data not shown). The relative molecular mass of this band coincided with the value calculated from the deduced amino-acid sequence, indicating



DED or DRD motifs⁴⁶ are underlined. Numbers in parentheses on the left indicate the positions of the first and last residues in the proteins. **c**, CED-4-homologous domain of FLASH was aligned with the nucleotide-binding and oligomerizing domains of CED-4, Apaf-1, Prf and RPP5 (refs 30, 47). The Walker's A- and B-box

consensus sequences for nucleotide-binding sites are underlined. **d**, FLASH and its deletion variants. CED-4- and DED-homologous regions, the Walker's A-box consensus motif for the nucleotide-binding site, and the putative NES and NLS are indicated. Numbers refer to amino-acid positions.

that we had isolated the full-length clone of mouse FLASH. The 4.7-kb messenger RNA could encode truncated FLASH, although we could not clone this cDNA by 5'-RACE PCR and colony hybridization. The significance of this mRNA remains to be determined.

FLASH interacts with caspase-8 and FADD

To investigate the interaction of FLASH with caspase-8 in mammalian cells, a Flag-tagged FLASH-expression plasmid (pME18S-Flag-FLASH) was used for transient transfection with plasmid for the protease-dead form of procaspase-8 with a histidine tag (pME18S-His-caspase-8(C360S)) in human 293T cells. His-procaspase-8(C360S) was specifically co-immunoprecipitated with Flag-FLASH by an anti-Flag monoclonal antibody (Fig. 3a). The FLASH immunoprecipitate contained the processed N-terminal fragment of caspase-8, p43 (His-p43). As FLASH bound the DED domain of FADD in yeast (data not shown), we tested whether transiently expressed FADD was co-immunoprecipitated with Flag-FLASH in 293T cells. Overexpressed haemagglutinin-tagged (HA)-FADD was found to co-immunoprecipitate with Flag-FLASH using anti-Flag mAb (Fig. 3c). FLASH was also shown to bind caspase-8 and FADD through its DED-like DRD domain:

DRD bound to caspase-8 and FADD whereas the CED-4-like domain and N-terminal region of FLASH could not bind to these proteins (Fig. 3b, d). Thus, overexpressed FLASH interacts with caspase-8 and FADD in mammalian cells.

FLASH is a component of DISC

We analysed the binding of transiently expressed FLASH to endogenous caspase-8, FADD and Fas before and after stimulation of Fas with the agonistic anti-Fas monoclonal antibody CH11 in 293Fas cells (Fig. 3e). Fas and FADD was mainly co-immunoprecipitated with Flag-FLASH only after stimulation of Fas, whereas caspase-8 likewise bound FLASH, regardless of whether Fas was stimulated. These results indicate that FADD and the complex of FLASH with caspase-8 might be recruited to Fas after stimulation of Fas, and that the association of FLASH and Fas might be indirect.

The formation of DISC, which is composed of at least Fas, FADD and caspase-8, is required for signal transduction of Fas-mediated apoptosis^{24,29}. We next tested whether endogenous FLASH could be a component of DISC. Mouse WR19L12a cells stably expressing human Fas were stimulated with CH11, and then Fas was immunoprecipitated with the anti-Fas monoclonal antibody HFE7A. Consistent with the results of our overexpression experiment,

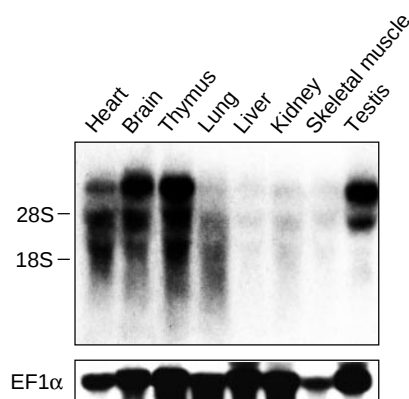


Figure 2 Tissue distribution of mouse FLASH mRNA. Northern blotting analysis has been described⁴³. Expression of EF1 α mRNA was used as a standard.

endogenous FLASH specifically co-immunoprecipitated with activated Fas (Fig. 4a, lane 3), indicating that FLASH is a component of DISC. The specificity of the anti-FLASH antibody was confirmed by western blotting of a total lysate from 293T cells transfected with or without Flag-FLASH, which showed a specific band at 220K (Fig. 4a, lanes 6 and 7). These observations indicate that this antibody can recognize not only transfected mouse FLASH but also human FLASH in 293T cells.

We then analysed DISC formation in two different human cell lines, SKW6.4 and Jurkat. SKW6.4 and Jurkat cells were reported to be type I and type II cells, respectively²⁴. In type I cells, Fas triggering caused strong caspase-8 activation at DISC; only a small amount of DISC was formed in type II cells. In type I SKW6.4 cells, DISC consisting of Fas, FADD, caspase-8 and FLASH was formed after a 5-min stimulation of Fas (Fig. 4b). There was a weak interaction between Fas and FADD before Fas stimulation, but none between caspase-8 and Fas. In type II Jurkat cells, which express comparable amounts of DISC components, including FLASH, DISC formation occurred after 5 min of Fas stimulation, although there was much less FADD, FLASH and caspase-8 co-immunoprecipitating with Fas than in type I cells. These results indicate that FLASH is a component of DISC in both type I and type II cells.

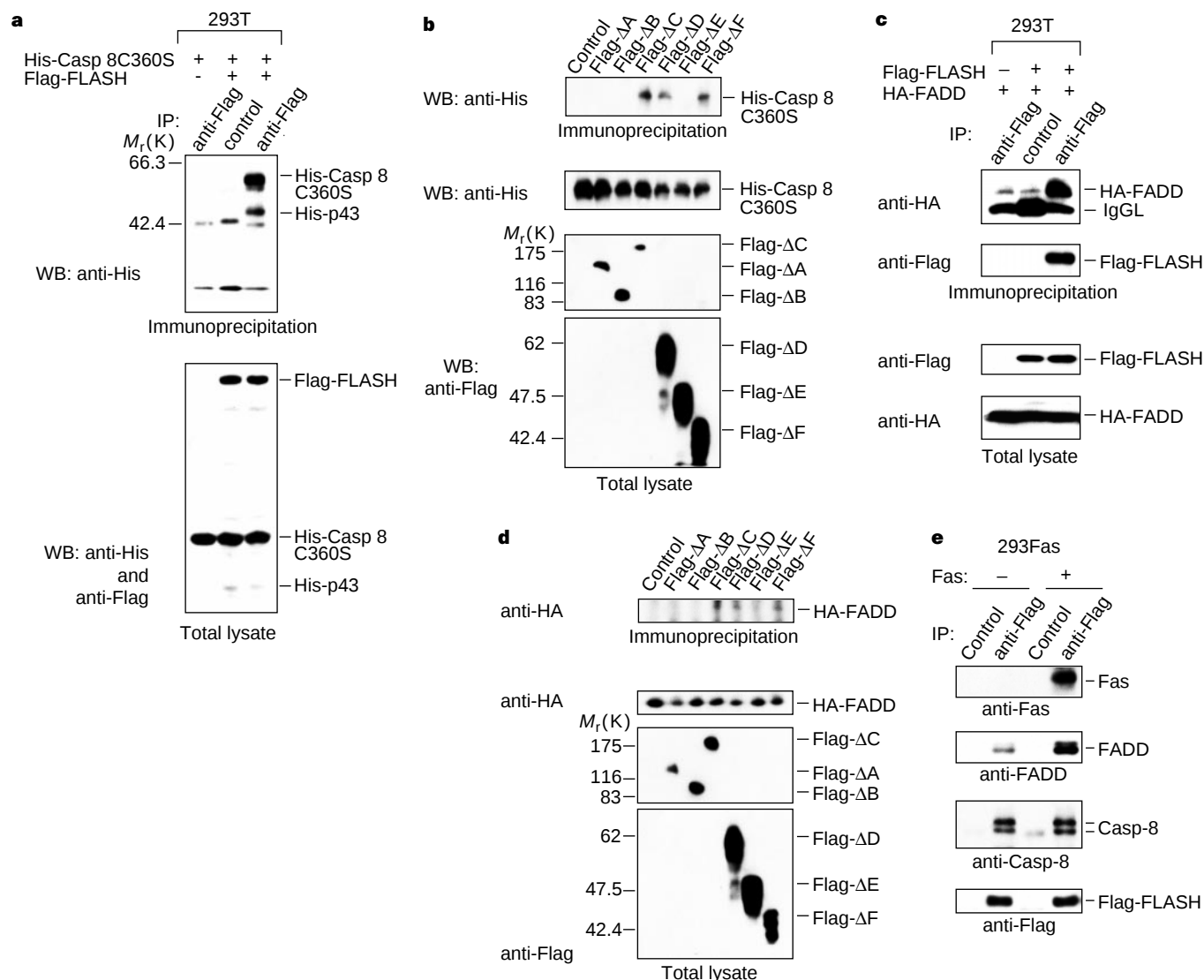


Figure 3 Overexpressed FLASH interacts with caspase-8, FADD and Fas in mammalian cells. Interaction of FLASH and its deletion variants with caspase-8 (a and b, respectively) and FADD (c and d, respectively). e, Interaction of

transiently expressed FLASH with endogenous Fas, FADD and caspase-8 in the presence or absence of Fas stimulation. See Methods. IP, immunoprecipitation; WB, western blotting.

Activation of caspase-8 requires FLASH

To investigate the function of FLASH, we analysed the effects of transiently transfected FLASH and its deletion mutant on activation of endogenous caspase-8 by processing in 293Fas cells (Fig. 5a). Expression of FLASH and its mutant was confirmed by western blotting with anti-Flag monoclonal antibody (Fig. 5b, lanes 2 and 3). Immunoblotting with anti-caspase-8 showed that the active p18 subunit of endogenous caspase-8 appeared 120 min after stimulation of Fas in control 293Fas cells. The p18 subunit appeared sooner (30 min after stimulation) in 293Fas cells expressing transfected wild-type FLASH. Overexpression of the truncated ΔD mutant of FLASH (comprising amino acids 1,553–1,962; Fig. 1d), which includes the DRD domain, significantly attenuated the production of p18, although the p43-cleaved intermediate of caspase-8 still appeared in cells transfected with ΔD , as it did with intact FLASH.

We next analysed whether transiently overexpressed FLASH and its mutants (Fig. 1d) could affect Fas-mediated apoptosis in HeLa-cell-derived HF1 cells stably expressing transfected human Fas (Fig. 5c, f). Overexpression of wild-type FLASH or the ΔC FLASH mutant in HF1 cells potentiated Fas-mediated apoptosis after stimulation with anti-Fas antibody, although transfected FLASH and ΔC gave no significant effects in the absence of Fas signalling. In contrast, overexpression of either the ΔB or ΔD mutant inhibited Fas-mediated apoptosis, with co-transfection of ΔB and ΔD giving the strongest inhibition. Results were similar when 293Fas cells were used instead of HF1 cells (data not shown). In addition, the effects of overexpressed FLASH and its mutants were essentially the same in TNF-mediated apoptosis on HF1 cells, whereas FLASH did not significantly affect staurosporine (STS)-induced apoptosis (Fig. 5d, e). Taken together, these results indicate that FLASH, which is recruited to Fas together with caspase-8 upon stimulation of Fas, is involved in Fas- and TNF-induced apoptosis mediated by activated caspase-8, although not in staurosporine-mediated apoptosis that may not need caspase-8. In addition, both the DED-like DRD and CED-4-like domains of FLASH are important in Fas- and TNF-induced apoptosis.

Self-association of FLASH

In *C. elegans*, CED-4 processes CED-3 by oligomerization, which is inhibited by binding of CED-9 or Bcl-X_L to CED-4 (ref. 30). These observations raised the possibility that FLASH might activate caspase-8 in the same way, so we tested whether FLASH could self-associate *in vivo*. We transfected Flag-tagged FLASH with or without Myc-tagged FLASH into 293T cells, and then immunoprecipitated it with anti-Flag antibody; we found that Myc-FLASH immunoprecipitated with Flag-FLASH (Fig. 6a). Next, four Flag-tagged deletion mutants of FLASH were co-expressed with full-length Myc-FLASH to identify the domain through which FLASH self-associates. Myc-FLASH co-immunoprecipitated with deletion mutant ΔB , which contains the CED-4-homologous domain, and with ΔC , which has the CED-4-homologous domain and the DRD domain, but not with the N-terminal ΔA mutant, which lacks both, or with the C-terminal ΔD mutant containing DRD (Fig. 6b). These results indicated that FLASH self-associates through the CED-4-homologous domain.

Interaction of FLASH with E1B19K

Although the anti-apoptotic adenovirus protein E1B19K suppresses the activation of caspase-8 during Fas-mediated and FADD-induced apoptosis²³, very little is known about how it does this. We therefore tested whether FLASH interacts with E1B19K by overexpressing E1B19K with Flag-FLASH in 293T cells. Flag-FLASH was immunoprecipitated with anti-Flag and the co-immunoprecipitated E1B19K was analysed by western blotting (Fig. 7a). We then analysed which domain of FLASH was interacting with E1B19K in 293T cells using our mutants ΔA – ΔF and found that the

C-terminal region of FLASH (ΔD), including the DRD domain, could interact with E1B19K (data not shown).

FLASH attenuates the function of E1B19K

We next tested the effects of FLASH on Fas-mediated apoptosis in FH2 cells transfected with E1B19K and also compared the effects of FLASH on the anti-apoptotic activity of E1B19K and on Z-VAD-fmk and poxvirus serpin CrmA, which are direct inhibitors of caspase-8 (Fig. 7b). We found that anti-Fas-induced apoptosis was blocked not only by pretreatment with Z-VAD-fmk but also by overexpression of CrmA or E1B19K. Although transiently expressed FLASH did not significantly stimulate Fas-mediated apoptosis in Z-VAD-fmk-treated or CrmA-expressing cells, it significantly inhibited the anti-apoptotic activity of E1B19K in Fas-mediated apoptosis. Essentially similar results were obtained

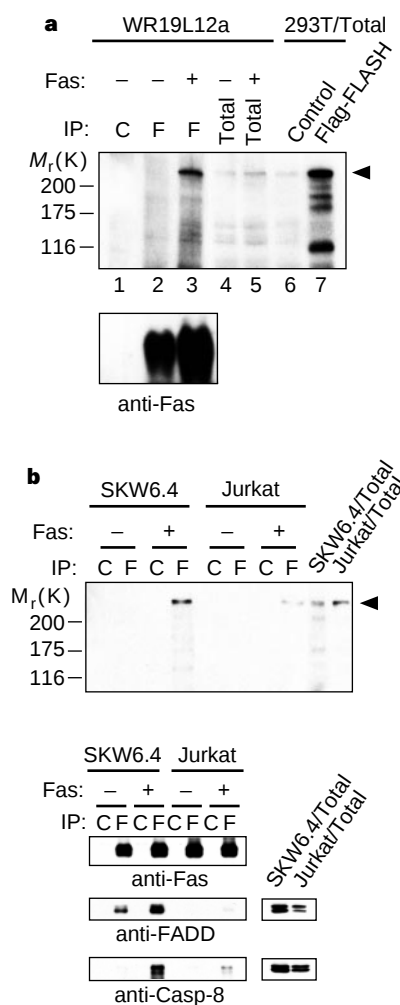


Figure 4 Endogenous FLASH was recruited to activated Fas in mouse WR19L12a and human SKW6.4 and Jurkat cells. **a**, **b**, Cells (WR19L12a for **a** and SKW6.4 and Jurkat for **b**) were treated with CH11, and immunoprecipitation was performed with HFE7A (F) or control antibody (C). Co-immunoprecipitated endogenous FLASH was detected by western blotting using rabbit anti-FLASH antibody, and immunoprecipitated Fas was also detected using anti-Fas mAb ZB4 (ref. 48) (bottom; lanes 1–3). In **a**, total lysates of the following cells were analysed by immunoblotting with anti-FLASH antibody: Fas-stimulated (lane 4) and unstimulated (lane 5) WR19L12a cells, and Flag-FLASH-expressing (lane 7) and control (lanes 6) 293T cells. In **b**, co-immunoprecipitated FADD and caspase-8 in SKW6.4 and Jurkat cells were detected using anti-FADD and anti-caspase-8 monoclonal antibodies. Total lysates of SKW6.4 and Jurkat cells were analysed by immunoblotting with anti-FLASH antibody, anti-FADD and anti-caspase-8 monoclonal antibodies. Arrowheads indicate the position of FLASH.

when HF1 cells were used instead of FH2 cells, indicating that FLASH links caspase-8 to E1B19K and E1B19K inhibits the activation of caspase-8 by binding to FLASH.

Discussion

Although FLASH associates with Fas and FADD as well as procaspase-8 in mammalian cells (Figs 3, 4), we do not know whether the association is direct or indirect, or with which protein(s) FLASH prefers to interact under physiological conditions. On the basis of our results from co-immunoprecipitation with and without Fas (Figs 3, 4), FLASH seems to be associated with procaspase-8 in non-apoptotic mammalian cells. Once Fas signalling is stimulated, FADD and then the FLASH–procaspase-8 complex are recruited to the intracellular domain of aggregated Fas at the cellular membrane in both SKW6.4 and Jurkat cells (previously reported as type I and type II cells²⁴, respectively) (Fig. 4). FLASH may promote the activation of caspase-8 in DISC with the help of FADD, given that the FLASH mutants ΔB and ΔD inhibit Fas-mediated activation of caspase-8 and apoptosis.

Because of the hydrophobicity in the prodomain of procaspase-8, this protein is likely to aggregate (Y.I. *et al.*, unpublished observations) and be auto-activated in the cytosol. Overexpression of procaspase-8 or of the DED domains of caspase-8 can induce apoptosis. Under physiological conditions, however, the concentra-

tion of procaspase-8 may be too low for it to aggregate and enable DISC to be activated rapidly. CED-4 and Apaf-1, respectively, have been proposed to serve as chaperones to control the local concentrations of CED-3 and procaspase-9 and to facilitate their autocatalytic activation by inducing conformational changes or oligomerization in response to upstream apoptotic signals. These effects of CED-4 and Apaf-1 are mediated by homophilic interaction between the so-called CARD domains^{30,31}. We propose that FLASH may serve as a chaperone to control the conformation and local concentration of procaspase-8 before the stimulation of Fas; also, FLASH may promote the autocatalytic activity of procaspase-8 with the help of FADD by binding to procaspase-8 through DRD–DED interaction and oligomerizing procaspase-8 through its CED-4-like domain after activation of Fas (Fig. 6).

FLASH mutants ΔB and ΔD inhibit Fas-mediated apoptosis in HF1 (Fig. 5c, f) and 293Fas cells (data not shown); they also inhibit TNF-mediated but not staurosporine-induced apoptosis, indicating that FLASH must be required for activation of caspase-8. The binding of ΔD to procaspase-8 (Fig. 3b) could prevent it from binding to endogenous FLASH, and ΔB may inhibit the oligomerization of endogenous FLASH by binding to it (Fig. 6), so a combination of ΔB and ΔD caused the strongest inhibition of Fas- and TNF-mediated apoptosis.

More FLASH messenger RNA was expressed in thymocytes (type

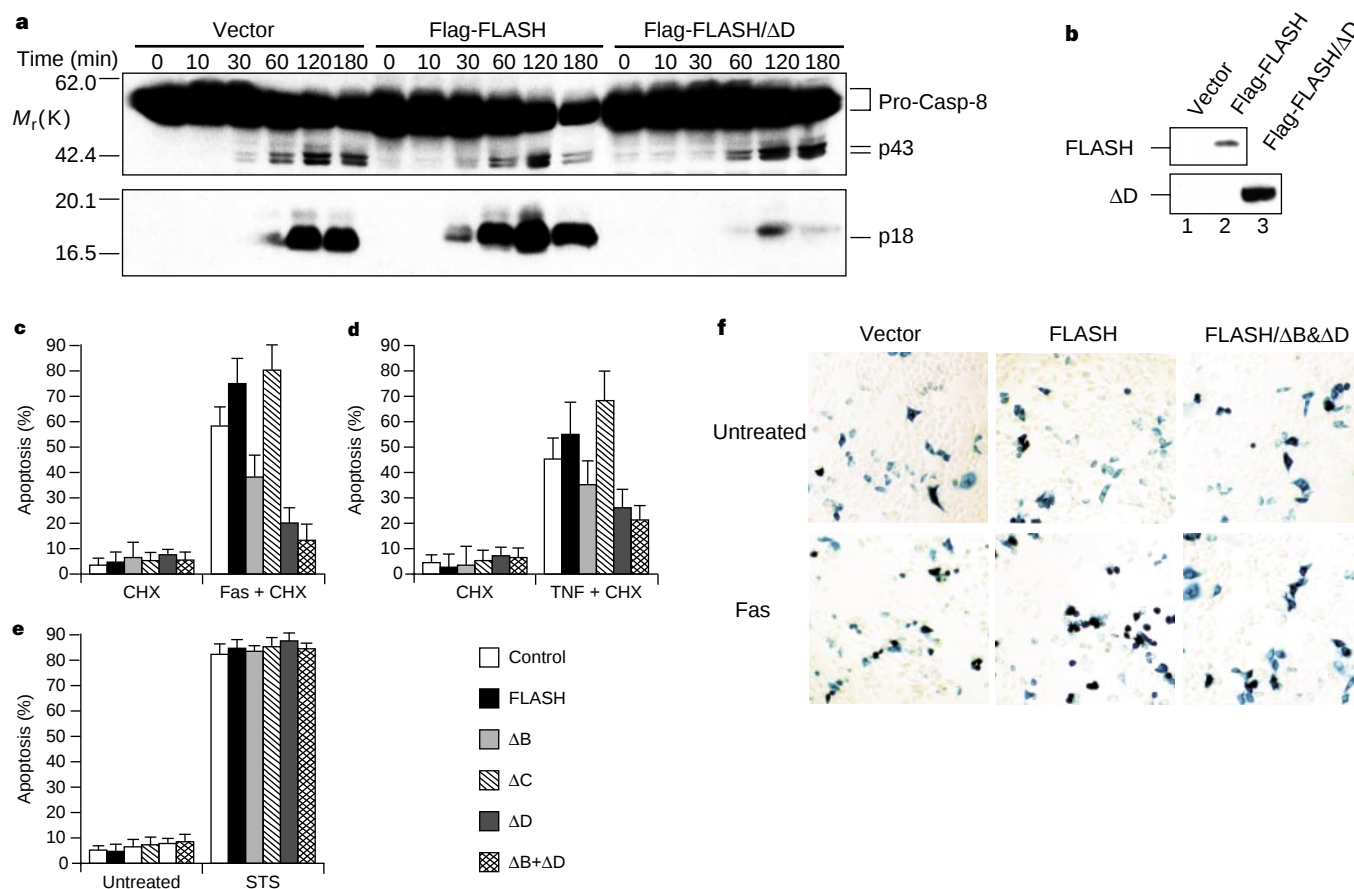


Figure 5 Effects of transfected FLASH and its mutants on activation of caspase-8 and Fas-induced apoptosis. **a**, Effects of FLASH on cleavage-associated activation of caspase-8. Human 293Fas cells were transiently transfected with pME18S, or pME18S-Flag-FLASH or -FLASH/ ΔD , and then treated with CH11 (2 $\mu\text{g ml}^{-1}$) for the indicated times. Total lysates from 10^6 cells were analysed by immunoblotting with anti-caspase-8 mAb. Migration positions of full-length (Pro-Casp-8), cleaved intermediate (p43; doublet) and the activated large subunit (p18) of caspase-8 are indicated. **b**, Expression of transfected FLASH and its mutants in 293Fas cells. Total cellular lysates of 293Fas cells (10^6 cells per sample) transiently

transfected with pME18S (lane 1), or pME18S-Flag-FLASH (lane 2), or pME18S-Flag-FLASH/ ΔD (lane 3) were immunoblotted using anti-Flag mAb. **c–e**, Effects of FLASH on apoptosis mediated by anti-Fas antibody CH11 (**c**), TNF (**d**) and STS (**e**). HF1 cells were transfected with control vector, Flag-FLASH-encoding vector, or vectors harbouring deletion mutants of FLASH together with pJ7-LacZ, and were then treated with CH-11 (0.1 $\mu\text{g ml}^{-1}$) and cycloheximide (10 $\mu\text{g ml}^{-1}$), TNF (0.1 $\mu\text{g ml}^{-1}$) and cycloheximide (10 $\mu\text{g ml}^{-1}$) or STS (1 μM) for 6 h. **f**, Morphology of the stained cells in **c**. Cells transfected with the indicated plasmids were treated either with (lower) or without (upper) CH11. CHX, cycloheximide.

I cells) than in hepatocyte (type II cells) (Fig. 2). The difference between type I and type II cells²⁴, however, could not be explained by endogenous expression of caspase-8 and FLASH, given that the expression of these proteins is similar in type I SKW6.4 cells and in type II Jurkat cells, although there is slightly less FADD in the latter (Fig. 4b). Recruitment of FADD to Fas after and even before stimulation of Fas is more likely in type I SKW6.4 cells than in type II Jurkat cells (Fig. 4b). These results suggest that recruitment of FADD can cause sufficient DISC formation in type I cells, although it is possible that recruitment of the FLASH–caspase-8 complex to Fas–FADD complex is dominant.

We found that a portion of FLASH transiently transfected into mammalian cells could be proteolytically cleaved without Fas stimulation (Fig. 4a, lane 7). We suspected that overexpressed FLASH could be processed by caspase-8 activated in a Fas-independent manner, because the cleaved products of FLASH disappeared from cells treated with the caspase inhibitor Z-VAD-fmk (a general caspase inhibitor) or Z-IETD-fmk (a caspase-8-like caspase inhibitor), but not Ac-DEVD-CHO (a caspase-3-like caspase inhibitor) (data not shown). There are potential cleavage sites for caspase in FLASH and putative NLSs are present in the C-terminal region, suggesting that cleaved C-terminal FLASH with the DRD domain might translocate to the nucleus following caspase activation. The cleavage products of FLASH may have a role in caspase-dependent nuclear translocation of apoptotic effectors or signal amplifiers such as CAD/DFF40 and DEDD (refs 20, 32, 33). Alternatively, the processing of FLASH may be a negative-feedback pathway for activating caspase.

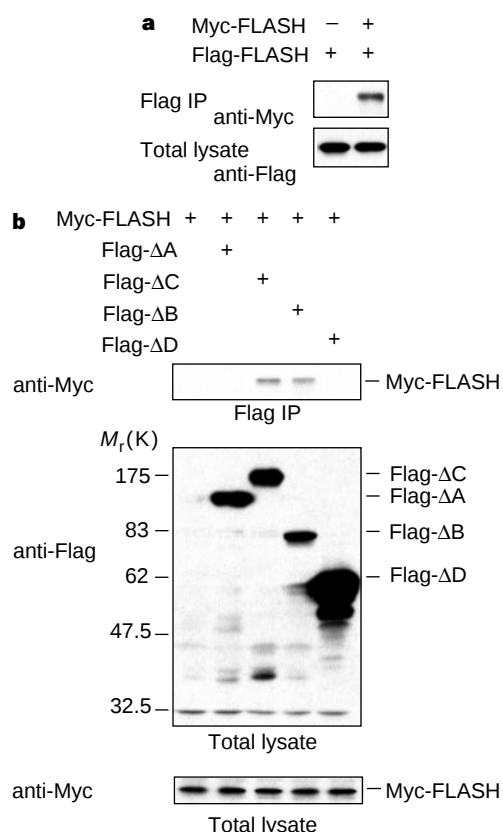


Figure 6 Self-association of FLASH through its CED-4-homologous domain. **a**, **b**, Myc-FLASH was expressed in 293T cells with Flag-FLASH (**a**) or various Flag-tagged deletion mutants of FLASH (see Fig. 1d) (**b**) in the presence of 20 μ M Z-VAD-fmk. Myc-FLASH, co-immunoprecipitated with Flag-FLASH or Flag-tagged deletion mutants using anti-Flag mAb, was detected with anti-Myc mAb 9E10. Expression of Flag-FLASH and Myc-FLASH was determined by western blotting for total-cell lysate with anti-Flag and anti-Myc mAbs, respectively.

FLASH has a Walker's A-box motif for ATP/GTP binding in the CED-4-like domain and is specifically labelled *in vitro* by 5'-*p*-fluorosulphonylbenzoyl adenosine, an ATP analogue (data not shown). FLASH has an extra 24 amino acids between the Walker's A and B boxes compared with other CED-4-related molecules. In an experiment in which the FLASH ATP/GTP-binding site was disrupted (using an A-box K950R mutant), there was no significant activation of caspase-8 or apoptosis (data not shown). It is hard to assess the dominant-negative effect of the mutant as both the mutant and full-length FLASH were poorly expressed. The ATP/GTP requirement of FLASH *in vivo* therefore remains to be determined.

CED-4 and its mammalian counterpart Apaf-1 activate procaspases by oligomerization through the CED-4 domain, an effect of that is inhibited by binding of CED-9 and the anti-apoptotic Bcl-2 family of proteins, respectively^{30,31}. We have shown that FLASH

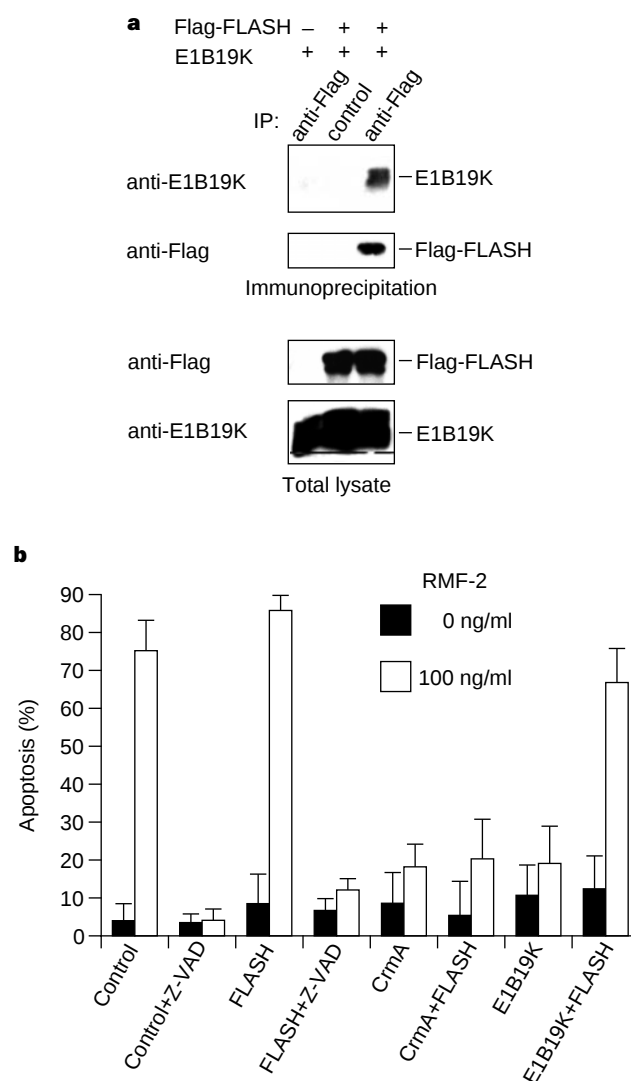


Figure 7 FLASH attenuates the anti-apoptotic effect of adenovirus protein E1B19K. **a**, Adenovirus E1B19K was expressed in 293T cells with or without Flag-FLASH in the presence of 20 μ M Z-VAD-fmk. Cell lysates were prepared as for Fig. 3a. Co-immunoprecipitated E1B19K was detected by western blotting using anti-E1B19K serum. Expression of Flag-FLASH and E1B19K was determined by western blotting of the total cell lysate with anti-Flag and anti-E1B19K, respectively. **b**, FH2 cells were transfected with control vector, or expression vectors for CrmA or E1B19K together with Flag-FLASH expression vector or empty vector. Apoptosis was assayed as described in Methods. For analysis using the synthetic peptide inhibitor Z-VAD-fmk (Z-VAD), cells were incubated with 20 μ M Z-VAD-fmk from 30 min before to 6 h after Fas stimulation.

self-associates through its CED-4-homologous domain in the same way as CED-4 and Apaf-1 (Fig. 6). FLASH binds E1B19K, another member of the Bcl-2 family, preventing it from inhibiting Fas-mediated apoptosis (Fig. 7). As E1B19K prevents apoptosis induced by FADD as well as by Fas, but not by caspase-8 (ref. 23), it probably works before caspase-8 is activated and mitochondrial damage is induced by Bid^{34–36}. E1B19K could block formation of the DISC complex by binding to FLASH, and overexpressed FLASH might stop E1B19K binding to the complex of FLASH with caspase-8. FLASH and Bcl-2, both of which are overexpressed in 293T cells, can be co-immunoprecipitated (data not shown), but the significance of this *in vivo* is unclear as Bcl-2 does not attenuate Fas-mediated activation of caspase-8 in type I cells²⁴. In type II cells, Bcl-2 may inhibit Fas-mediated apoptosis²⁴, not only by suppressing Bid-induced mitochondrial damage after caspase-8 is activated in the DISC complex^{34–36}, but also by inhibiting the activation of caspase-8 as a result of binding to the FLASH–procaspase-8 complex.

In *C. elegans*, CED-4 binds to both CED-3 and CED-9 in a complex called the apoptosome^{37,38}; CED-9 inhibits oligomerization of CED-4 and hence apoptosis³⁰. In mammalian systems, CED-4-homologous Apaf-1 binds to procaspase-9 and cytochrome *c* (ref. 22); Bcl-2 prevents release of cytochrome *c* from mitochondria^{39,40} and may bind to Apaf-1 and inhibit it^{41,42}. The mechanisms of inhibition by CED-9 and Bcl-2 of CED-4 and Apaf-1, respectively, are therefore different, and in this respect FLASH, which can bind to pro-caspase-8 and to Bcl-2 family proteins, is like CED-4. Apoptosomes may be genetically conserved in the FLASH system, but the physiological significance of the interaction between FLASH and the Bcl-2 family of proteins is unclear. In conclusion, our results indicate that FLASH has a similar function to CED-4, oligomerizing like CED-4 or Apaf-1 in order to activate procaspase-8. □

Methods

cDNAs, cDNA libraries and antibodies. cDNA fragment of mouse caspase-8 encoding amino-acid residues 1–202 was generated by PCR using pME18S–caspase-8 (ref. 43) as template and was cloned into the yeast expression vector pGBT9 (Clontech). Human caspase-8 cDNA (C360S) with a histidine tag was generated by using a Quick-change site-directed mutagenesis kit (Stratagene) and His-tagged human caspase-8 cDNA, prepared by conventional RT-PCR, as a template and then cloned into the mammalian expression vector pME18S (ref. 44). The random oligonucleotide- and oligo(dT)-primed cDNA libraries for yeast expression screening were prepared from poly(A)⁺ RNA of the mouse T-cell lymphoma cell line WR19L12a (ref. 1) and the mouse embryonal carcinoma cell line F9 using TimeSaver cDNA synthesis kits (Amersham–Pharmacia), and inserted into pGAD424.

Anti-human Fas mAb (HFE7A) was freshly prepared. Rabbit polyclonal antibody against mouse FLASH was raised against a 13-mer peptide, LSPNSDRNGDAHR, of FLASH (amino-acid residues 1,843–1,855), and affinity-purified with a deletion mutant of Flag-tagged FLASH (Δ D:C-terminal residues 1,553–1,962).

cDNA cloning of FLASH. 5'-RACE PCR combined with nested PCR was performed using a cDNA library of WR19L12a cells in pGAD424 as the template. cDNA libraries of F9 cells were screened using 5'-RACE PCR products encoding amino acids 1–542 and 586–1,012 of FLASH as probes by standard colony hybridization procedures⁴⁵. Five independent clones were sequenced, revealing that these overlapping clones covered the entire coding region of FLASH. Full-length mouse FLASH cDNA was obtained by ligating these clones. cDNA encoding full-length and various deletion mutants of mouse FLASH were cloned into the mammalian expression vector pME18S–Flag2, or pME18S–Myc.

Cell lines, transfection and assay of Fas-mediated apoptosis. Human 293 cells stably expressing human Fas (293Fas) and mouse Balb3T3 cell-derived FH2 cells stably expressing transfected mouse Fas were prepared in this laboratory. Cells precultured for 1 day were transfected with various expression vectors using the LipofectAMINE PLUS Reagent (Life Technologies) or cells were transfected by electroporation at 300 volts with a capacitance of 960 μ F using a Gene Pulser (Bio-Rad). Total amounts of transfected plasmid DNA in

individual experiments were adjusted to be the same with the empty vector plasmid. Transfected cells were cultured for at least 24 h after transfection and were used for immunoprecipitation or induction of Fas-mediated apoptosis.

Human HF1 cells and mouse FH2 cells were transfected with various expression vectors, together with the β -galactosidase expression vector pJ7–LacZ. After 24 h cultivation, HF1 and FH2 cells were stimulated with the human anti-Fas antibody CH11 (ref. 3) in the presence of 10 μ g ml^{−1} cycloheximide and anti-mouse Fas monoclonal antibody RFM-2 (ref. 45), respectively. Following incubation for 6 h, cells were fixed with 0.2% glutaraldehyde and 2% formaldehyde, and stained with X-Gal for 0.5–1 h. The numbers of blue β -galactosidase-positive cells with flat adherent morphology and with shrunken round morphology were counted as viable and apoptotic cells, respectively. At least 300 β -galactosidase-positive cells were scored for each transfection in triplicate, and the mean percentages of apoptotic blue cells and their standard errors were calculated.

Immunoprecipitation and western blotting analysis. Cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100, 1 mM PMSF, 2 μ g ml^{−1} aprotinin, 2 μ g ml^{−1} leupeptin and 2 μ g ml^{−1} pepstatin A). Flag-tagged FLASH was immunoprecipitated from the cell lysate with anti-Flag antibody and protein-G-coupled Sepharose beads (Amersham–Pharmacia), and washed four times with lysis buffer.

Immunoprecipitates or total cell lysates were analysed by western blotting with primary antibodies and horseradish-peroxidase-conjugated second antibodies, and specific bands were detected using chemiluminescence detection reagents (Renaissance; NEN Life Science). To assay the interaction between endogenous FLASH and Fas, WR19L12a cells (2×10^8) were treated with 2 μ g ml^{−1} CH11 for 15 min at 37 °C (stimulated sample) or for 30 min at 4 °C (unstimulated sample). In SKW6.4 and Jurkat cells (3×10^9), cells (60 ml) were treated with 2 μ g ml^{−1} CH11 for 5 min at 37 °C (stimulated sample) or for 30 min at 4 °C (unstimulated sample). Treated cells were lysed and immunoprecipitated with anti-human Fas antibody (HFE7A).

For Fig. 3, pME18S–His–caspase-8 (C360S) (His–Casp 8(C360S)) or pME18S–HA–FADD was introduced into 293T cells, together with pME18S encoding Flag–FLASH or Flag-tagged deletion mutants of FLASH (Fig. 1d), or empty pME18S. Transfected cells were cultured for 20 h and then for 16 h in the presence of 20 μ M Z-VAD-fmk (Peptide Institute, Osaka, Japan). Flag–FLASH was immunoprecipitated with anti-Flag mAb M2 (Kodak) from cell lysates containing 1 μ M Z-VAD-fmk. Co-immunoprecipitated His–Casp 8(C360S) and HA–FADD was detected using anti-His mAb^{RGS}–His (Qiagen) and anti-HA mAb 12CA5 (Roche Diagnostics), respectively. Total cell lysate was also analysed by western blotting using anti-Flag, anti-His and anti-HA monoclonal antibodies. 293Fas cells were transfected with pME18S–Flag–FLASH. 36 h later, cells were treated with or without 2 μ g ml^{−1} agonistic anti-human Fas mAb CH11 for 15 min. Then, Flag–FLASH was immunoprecipitated from cell lysates using anti-Flag mAb. Co-immunoprecipitated Fas, FADD and caspase-8 were detected by western blotting analysis using anti-Fas HFE7A, anti-FADD mAb clone 1 (Transduction Labs) and anti-caspase-8 mAb 5F7 (MBL, Nagoya, Japan). Immunoprecipitated Flag–FLASH was also detected with anti-Flag mAb.

Received 16 December 1998; accepted 26 March 1999.

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Acknowledgements. We thank Y. Tsujimoto for cDNAs encoding Bcl-2 and SKW6.4 cells, D. V. Goeddel for mouse FADD cDNA, D. J. Pickup for CrmA cDNA, S. Hashimoto for antiserum against E1B19K, S. Tsukumo for pME18S-Flag2, H. Kazama for pME18S-Myc and FH2 cells, and Y. Nakanishi for HF1 cells. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and by the Ministry of Health and Welfare, Japan.

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Josephson-quasiparticle (JQP) current^{16,17} and is carried by a cyclic process consisting of one Cooper-pair tunnelling between the two charge states and two sequential quasiparticle tunnelling events at the probe junction. When applying a pulse array (solid line), on the left side of the JQP peak we observed a pulse-induced current with several peaks whose positions did not depend on T_r but on Δt . In Fig. 3a we extract the pulse-induced part of the current, ΔI , for the pulse length $80 \leq \Delta t \leq 450$ ps. With increasing Δt , all the peaks moved towards smaller Q_0 and disappeared at $Q_0/e \approx 0.5$. The region where the peaks existed extended to smaller Q_0 linearly with increasing pulse height (data not shown).

We simulated the pulse operation of the quantum state by numerically solving a time-dependent Schrödinger equation. We

calculated the average increase in the probability density at $|2\rangle$ after a single-pulse operation, $\langle \Delta P(2) \rangle$, which should approximately be proportional to ΔI . To adjust the maximum oscillation period in the time domain, T_{coh} , we used a Josephson energy $E_J = 51.8 \mu\text{eV}$, and to adjust $Q_0 (= 0.51e)$ where T_{coh} was observed we used an effective pulse height $\Delta Q_p/e$ of 0.49. The overall features of the pulse-induced current were reproduced (Fig. 3b). The value of Q_0 where T_{coh} was observed corresponded to the point where the applied pulse brought the two levels into resonance and T_{coh} equalled h/E_J . The oscillation period in the time domain changed according to $h/\sqrt{E_J^2 + \delta E^2}$, where $\delta E \equiv 4E_C[(Q_0 + \Delta Q_p)/e - 1]$ is the electrostatic energy difference between the two charge states during the pulse.

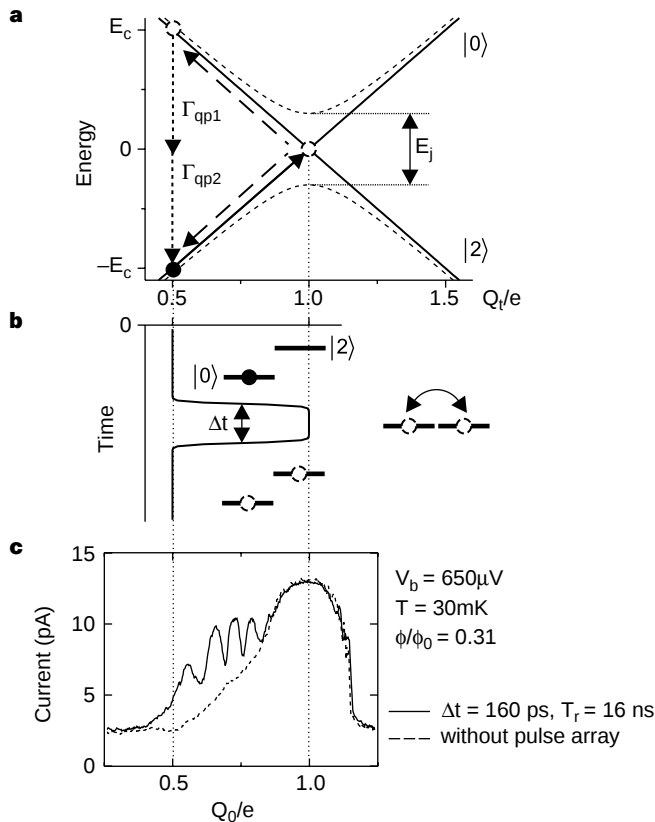


Figure 2 Pulse modulation of quantum states. **a**, Energy diagram illustrating electrostatic energies (solid lines) of two charge states $|0\rangle$ and $|2\rangle$ (with the number of excess charges in the box $n = 0$ and 2) as a function of the total gate-induced charge $Q_t = Q_0 + C_p V_p(t)$, where $Q_0 = C_g V_g + C_b V_b$ is the d.c.-gate induced charge. The dashed curves show eigenenergies (in the absence of the quasiparticle tunnelling at the probe junction) as a function of Q_t . Suppose that before a pulse occurs, Q_t equals Q_0 , which is far from the resonance point, and the system is approximately in the pure charge state $|0\rangle$ (filled circle at lower left). Then, a voltage pulse of an appropriate height abruptly brings the system into resonance $Q_t/e = 1$ (solid arrow), and the state starts to oscillate between the two charge states. At the end of the pulse, the system returns to $Q_t = Q_0$ (dashed arrow) with a final state corresponding to the result of the time evolution. Finally, the $|2\rangle$ state decays to $|0\rangle$ with two quasiparticle tunnelling events through the probe junction with rates of Γ_{qp1} and Γ_{qp2} (dotted arrows). **b**, Schematic pulse shape with a nominal pulse length Δt (solid line). The rise/fall times of the actual voltage pulse was about 30–40 ps at the top of the cryostat. The voltage pulse was transmitted through a silver-plated Be-Cu coaxial cable (above 4.2 K), a Nb coaxial cable (below 4.2 K) and an on-chip coplanar line to the open-ended pulse gate shown in Fig. 1a. The insets illustrate situations of the energy levels before/during/after the pulse. **c**, Current through the probe junction versus Q_0 with (solid line) and without (dashed line) the pulse array. The pulse length was $\Delta t = 160$ ps and the repetition time was $T_r = 16$ ns. The data were taken at $V_b = 650 \mu\text{V}$ and $\phi/\phi_0 = 0.31$, where $\phi_0 = h/2e$ is a flux quantum.

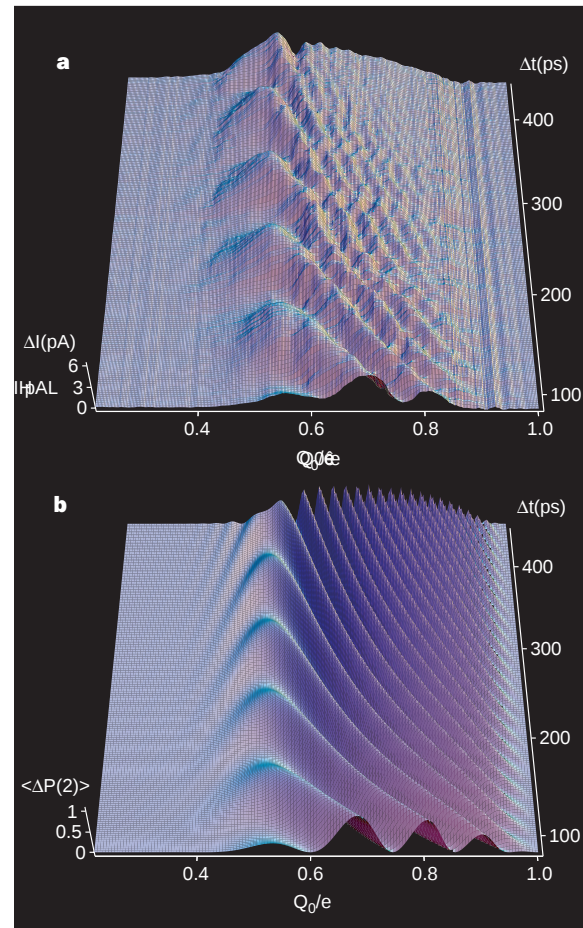


Figure 3 Effect of applying pulses as a function of d.c.-induced charge Q_0 and pulse length Δt . **a**, Three-dimensional plot of pulse-induced current ΔI which is the difference between currents measured with and without a pulse array. $\Delta t = 80$ ps was the shortest pulse length available with our pulse-pattern generator (Anritsu MP1758A). **b**, Calculated average increase in probability density at $|2\rangle$ after a single-pulse operation, $\langle \Delta P(2) \rangle$. The averaged probability density after the pulse was calculated by numerically solving a time-dependent Schrödinger equation and by averaging out small residual oscillations in the time domain. The effect of decoherence was not included. As the initial condition of the Schrödinger equation, we used a mixture of two eigenstates at $Q_t = Q_0$ with weights obtained from a steady-state solution of density-matrix equations that describe charge transport through the device in the absence of a pulse array. The initial probability density was also calculated from the steady-state solution. In the calculations, Josephson energy $E_J = 51.8 \mu\text{eV}$ and an effective pulse height $\Delta Q_p/e = 0.49$ were used. The solid line in Fig. 2b shows an example (at $\Delta t = 300$ ps) of the pulse shape used in this calculation.

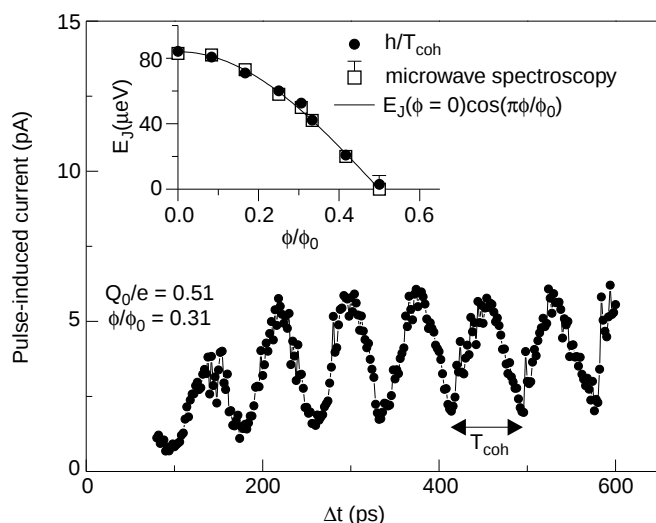


Figure 4 Pulse-induced current as a function of the pulse length Δt . The data correspond to the cross-section of Fig. 3a at $Q_0/e = 0.51$. Inset, Josephson energy E_J versus the magnetic flux ϕ penetrating through the loop. E_J was estimated by two independent methods. One was from the period of the coherent oscillation T_{coh} as h/T_{coh} . The other was from the gap energy observed in microwave spectroscopy⁴. The solid line shows a fitting curve with $E_J(\phi = 0) = 84 \mu\text{eV}$ assuming cosine ϕ -dependence of E_J .

Figure 4 shows the pulse-induced current at $Q_0/e = 0.51$ as a function of Δt , showing that the coherent oscillation can be observed in the time domain and that we can control the quantum state through an arbitrary pulse length Δt . The oscillation amplitude was smaller than that simply expected from $2e$ per pulse, $2e/T_r = 20 \text{ pA}$. The finite rise and fall times of the pulse might explain this deviation. We recall that in the limit of long rise and fall times (the adiabatic limit), there would be no transition probability to $|2\rangle$. For the realistic rise and fall times of the pulse we assumed in the simulation above, for example, the amplitude of the oscillations in $\langle \Delta P(2) \rangle$ at $Q_0/e = 0.51$ is reduced to ~ 0.4 , by which the current signal would be decreased. Moreover, the finite repetition time (not much longer than $\Gamma_{\text{qp1}}^{-1} + \Gamma_{\text{qp2}}^{-1}$) could also reduce the signal due to the incomplete relaxation of $|2\rangle$ to $|0\rangle$ after each pulse.

To further confirm that the observed oscillation was coherent oscillation due to Josephson coupling, we estimated the Josephson energy E_J from the oscillation period T_{coh} as $E_J = h/T_{\text{coh}}$ and investigated its magnetic-field dependence (filled circles in Fig. 4 inset). We also measured E_J in the frequency domain through microwave spectroscopy of the energy-level splitting⁴ (open squares in Fig. 4 inset). The two sets of data agreed very well, and fitted the expected cosine curve.

For future application as quantum computing devices^{5–7}, a crucial parameter is the decoherence time. The main decoherence source in a single-Cooper-pair box is thought to be spontaneous photon emission to the electromagnetic environment^{1,5–7}, and the decoherence time could exceed $1 \mu\text{s}$. But when a probe junction is used, as in our set-up, the ‘detection’ with quasiparticle tunnelling through the probe junction would be the main source of decoherence. So far, we have observed oscillation up to $\Delta t \approx 2 \text{ ns}$, although low-frequency background-charge fluctuation degraded the direct current signal and made it difficult to determine the envelope of the decay. A more detailed study of the decoherence time would provide important information for designing solid-state quantum circuits using superconducting single-Cooper-pair boxes. □

Received 26 January; accepted 18 March 1999.

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Acknowledgements. We thank W. Hattori, M. Baba and H. Suzuki for experimental help and M. Ueda, Y. Kohno, M. H. Devoret and Y. Ootuka for discussions. This work was supported by the Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation (JST).

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The existence of supercooled liquid water at 150 K

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Supercooled water may offer clues to the anomalous properties of its normal liquid state¹. The supercooled state also shows anomalous thermodynamic and transport properties at low temperatures^{2–4}. Although there are several theoretical explanations for this behaviour, no consensus has emerged^{1,2,5–12}. Some theories preclude the existence of the supercooled liquid below an apparent thermodynamic singularity at 228 K (refs 2, 7, 9); others are consistent with a continuous region of metastability from the melting point at 273 K to the glass transition temperature at 136 K (refs 6, 8, 13). But the data needed to distinguish between these possibilities have not yet been forthcoming. Here we determine the diffusivity of amorphous ice by studying isotope intermixing in films less than 500 nanometres thick. The magnitude and temperature dependence of the diffusivity is consistent with the idea that the amorphous solid water melts into a deeply metastable extension of normal liquid water before crystallizing at 160 K. This argues against the idea of a singularity in the supercooled regime at ambient pressure.

Water vapour deposited on low-temperature substrates ($< 140 \text{ K}$) is known to form an amorphous phase, termed amorphous solid water (ASW), that is metastable with respect to crystalline ice^{3,4,14}. There is still a debate about whether this amorphous form of water transforms to a metastable liquid above the glass transition temperature at 136 K and before crystallization near 160 K (refs 15–17). Furthermore, if the amorphous solid does melt into a liquid, a question remains as to whether this liquid is a metastable extension of supercooled liquid water or a distinct thermodynamic phase^{15–17}. We have recently measured the difference in the vapour pressure

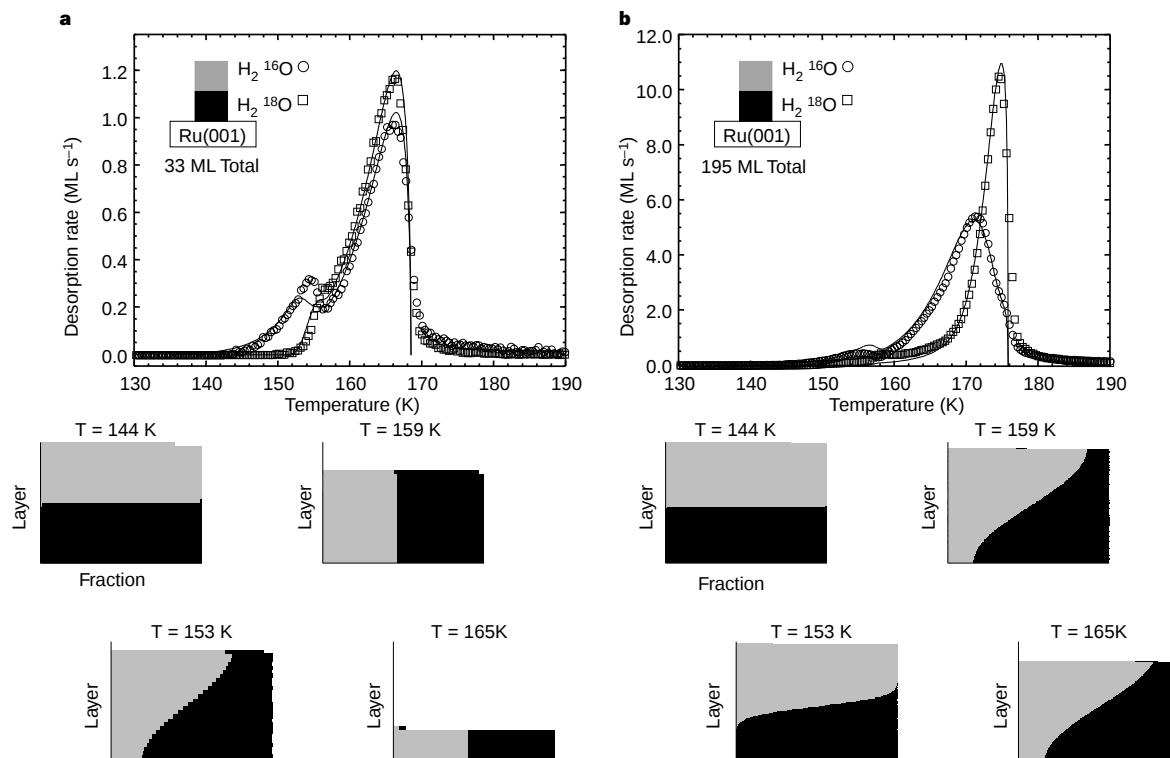


Figure 1 Comparison of TPD experiment and model simulation. The nanoscale films were created using an effusive molecular beam having a flux of ~ 0.06 monolayers per second, ML s^{-1} ($1 \text{ ML} = \sim 10^{15}$ molecules cm^{-2}). Water deposits were made at 85 K, and the temperature was then linearly ramped at a rate of 0.6 K s^{-1} . **a**, The TPD spectra (top) for an experiment where ~ 17 layers of H_2^{16}O (open circles) were deposited on ~ 16 layers of H_2^{18}O (open squares); the model simulation (solid line) is also shown. The experiments and simulation are in excellent agreement. The desorption data show that below 154 K only H_2^{16}O (the species on top) desorbs, but after the phase transition the desorption rates are nearly equal which indicates significant mixing. Bottom, the graphs below the TPD spectra are concentration profiles that reveal the layer-by-layer extent of mixing of the two isotopes at the given temperature. The mole fraction of each isotopic species (H_2^{16}O (light shade) and H_2^{18}O (dark shade)) within a given layer is represented as a horizontal bar graph. The profile at 144 K shows that the isotopes have not mixed, as the top layers consist of only the H_2^{16}O isotope while the bottom layers consist of only the H_2^{18}O isotope. But by 159 K the two isotopes have completely mixed, as is shown by the nearly equal amounts of the two isotopes in each layer. At higher temperatures the thin film has crystallized, so diffusion is 'frozen' and the concentration profile remains constant as the water

film desorbs. **b**, The TPD spectra (top) for an experiment where ~ 100 layers of H_2^{16}O (open circles) were deposited on ~ 95 layers of H_2^{18}O (open squares) to create a 195-ML-thick film; the model simulation (solid line) is also shown. The desorption data show that a large fraction of the H_2^{16}O (species on top) desorbs before the onset of significant H_2^{18}O (species on bottom) desorption. The concentration profiles (bottom) show that although at 153 K the films have begun to mix, at 159 K the two isotopes have not completely mixed before crystallization of the thin film. The distribution is effectively 'frozen' by the complete crystallization above 159 K. The use of thin water isotopic films to determine the extent of intermixing has been demonstrated previously¹⁸. The growth conditions used here produce optically flat non-porous water films^{19,37}. A roughened interface between the isotopic layers, which in the worst case would result in intermixing of $\sqrt{N}$ (where N is the number of layers), cannot account for the observed intermixing. The possibility of crack formation during the crystallization has also been studied²¹. Our kinetic model would not be able to reproduce the experimental data if the dominant intermixing mechanism were mixing along (or through) cracks formed in the films. Furthermore, such a mechanism could not account for the statistical isotopic exchange product yields shown previously¹⁸.

between ASW and crystalline ice and determined that thermodynamic continuity with normal liquid water is possible¹⁷. We have also shown that long-range molecular translational diffusion characteristic of liquid-like behaviour occurs before crystallization¹⁸. Estimates of the liquid diffusion coefficients yield a diffusivity roughly a million times greater than that of crystalline ice¹⁸.

We employ molecular beams of H_2^{16}O and H_2^{18}O with high spatial resolution to create nanoscale ASW films ($< 5,000 \text{ \AA}$ thick). Temperature-programmed desorption (TPD) is used to measure quantitatively the desorption kinetics, and thereby reveal the extent of mixing between the H_2^{16}O and H_2^{18}O layered interfaces^{17–21}. Quantitative measurements of the desorption rates from the amorphous and crystalline phases of H_2O and D_2O have been used to quantify the crystallization kinetics which can be described by a classic nucleation and growth mechanism^{17,20}. The conversion to the thermodynamically stable crystalline phase results in a concomitant decrease in the vapour pressure (desorption rate) and the irreversible amorphous to crystalline

transformation^{14,18,20,21} appears as a 'bump' in the 150–160 K temperature range of the TPD spectrum.

We quantify the temperature-dependent diffusivity using a mathematical model that couples our previous mean-field description of the desorption/crystallization kinetics²⁰ to a one-dimensional representation of the diffusive transport between layers. The effective diffusivity is a linear combination of the amorphous and crystalline diffusion coefficients weighted by their respective mole fractions. We assume an Arrhenius form for the temperature dependence of the diffusion coefficients, and vary the amorphous diffusion parameters to achieve a fit to the experimental data.

Figure 1 shows TPD spectra and model simulations for two different total thicknesses of ASW. As shown in Fig. 1a, the onset of isotopic mixing is abrupt and is concomitant with the phase transformation of ASW into crystalline ice. If the isotopically tailored layers did not exhibit diffusive intermixing, all of the H_2^{16}O molecules would desorb before the onset of H_2^{18}O desorption. This would be expected if the ASW phase had the diffusivity of

crystalline ice. The simulation results (solid lines) are in excellent agreement with the experiment. The four illustrations in the bottom of Fig. 1a show the vertical spatial distribution of the isotopes obtained from the simulation. At 144 K the two isotopes are vertically separated as deposited, but by 153 K significant intermixing has occurred. At 159 K the layers have completely mixed and the film has crystallized.

Figure 1b shows the experimental TPD spectra and the model simulation for a much thicker film. These spectra reveal that only a limited amount of mixing has occurred before complete crystallization. The vertical spatial distributions of the isotopes, illustrated in the bottom of the figure, show that mixing occurs in concert with the phase transition but that at 159 K the film has not mixed completely. Above 159 K the film is completely crystallized, and as such, the diffusive motion is 'frozen out'. As the film thickness increases, the films show departures from complete mixing because the effective mixing zone is limited by the time it takes for the film to crystallize. We have performed similar experiments for a variety of films, and find that the model simulation with a single set of amorphous diffusion parameters is in excellent agreement with the experimental data for thicknesses from 30 to 200 monolayers (ML) irrespective of which isotope is on top. Although not shown here, experiments using H/D labelled water reveal complete isotopic scrambling within the intermixed region, thereby demonstrating intimate chemical contact between the species¹⁸.

The temperature dependence of the diffusivity obtained from the

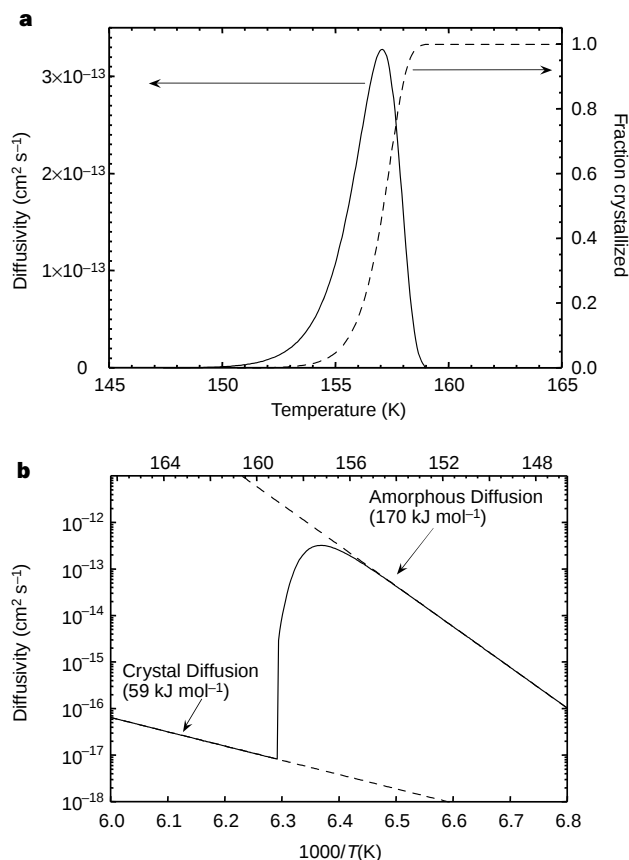


Figure 2 Diffusivity versus temperature from the model simulation of the TPD experimental data. **a**, The solid line is the diffusivity versus temperature, and the dashed line is the extent of crystallization of the ASW material. The maximum in the plot of diffusivity versus temperature is because the diffusivity in the amorphous liquid phase is much greater than that in the crystalline solid phase. **b**, An Arrhenius plot of the same diffusivity data (solid line). The dashed lines are Arrhenius fits to the pure amorphous and pure crystalline diffusion regions. The apparent activation energies for amorphous and crystalline diffusion are 170 and 59 kJ mol⁻¹, respectively.

simulation is shown in Fig. 2. In Fig. 2a the diffusivity (solid line) shows an initial exponential rise with temperature but reaches a maximum near 157 K and then drops rapidly. As the fraction crystallized^{17,18,20,21} increases from 0 to 1 (dashed line), the diffusivity transforms from completely amorphous diffusion to completely crystalline diffusion. As the diffusion in the amorphous liquid phase is much easier than in the crystalline solid phase, the effective diffusivity shows a maximum when plotted as a function of temperature. The diffusivity is shown in an Arrhenius plot in Fig. 2b. The low-temperature diffusivity data show that the amorphous diffusion has a relatively high activation energy. An Arrhenius fit (dashed line) to the diffusivity over the temperature range 147–157 K yields an apparent activation energy of 170 ± 40 kJ. The large uncertainty in the activation energy is due to the insensitivity of the simulations to the slope of the Arrhenius fit over the narrow temperature range before the onset of crystallization. Nonetheless, the simulations are extremely sensitive to $\pm 50\%$ variations in the absolute value of the amorphous diffusivity over this narrow temperature range. Above 157 K, the diffusivity drops rapidly owing to crystallization. Diffusion in crystalline ice in this temperature range (up to 170 K) is extremely small^{22,23}, and nearly identical simulation results are obtained when the value of crystalline diffusion is set to zero. Near 160 K the diffusivity of ASW reaches $\sim 10^{-12}$ cm² s⁻¹, roughly 10^7 times smaller than the diffusivity of normal liquid water at room temperature. Such a diffusivity would be nearly impossible to observe with a macroscopic sample—a 1-cm-thick film would require $\sim 10^5$ years to mix completely. The use of nanoscale films enables these small diffusivities to be determined quantitatively.

Figure 3 shows the temperature dependence of the diffusivity for ASW, liquid water²⁴, supercooled liquid water^{25,26}, and crystalline ice^{22,23}. An Arrhenius extrapolation of the liquid and supercooled liquid data does not agree with our ASW diffusivity results; our results show a much stronger temperature dependence, with an apparent activation energy of ~ 170 kJ mol⁻¹. It is known that glass-forming liquids show markedly non-Arrhenius behaviour as they are supercooled below their freezing point^{5,27}. The temperature dependence of this non-Arrhenius behaviour is often well represented by the empirical Vogel–Fulcher–Tammann (VFT) equation^{5,27}, $D = D_0 \exp[-E/(T - T_0)]$ where D is the temperature dependent diffusivity, T is the temperature, and D_0 , E and T_0 are fit parameters. The solid line labelled VFT in Fig. 3 is the result of fitting all of the diffusivity data to the VFT equation.

It has been proposed² that there is a thermodynamic singularity in the water phase diagram at 228 K. This proposal was partly based on the observation that many of the physical properties of supercooled water could be described by a power-law equation of the form, $A = A_0(T/T_s - 1)^\gamma$, where A is the property of interest and A_0 , γ and T_s are fit parameters. One interpretation of this singularity is given by the ‘stability limit conjecture’, which predicts that normal liquid water cannot exist below T_s , due to a retracing spinodal⁷. Another interpretation is that the apparent singularity is due to the existence of a second critical point in the water phase diagram^{8,12}. The power-law prediction for the liquid and supercooled-liquid diffusivity data is plotted in Fig. 3. Over the temperature range 250–500 K, a comparison of the fits does not provide unambiguous evidence in support of either the VFT or power-law equations. On an expanded scale (Fig. 3 inset), the VFT equation deviates from the liquid data below 250 K by about 25% and the power law gives a better fit. However, assuming that the diffusivities from the two temperature regions can be connected, then the VFT equation describes the entire set of data spanning a range of 10^{10} reasonably well (within a $\pm 25\%$ deviation). We note that the power-law equation, or any equation with a temperature singularity at 228 K, cannot fit both the ASW and the liquid diffusivity data.

The open question is whether there is a continuity between the normal supercooled-liquid diffusivity and the liquid-like diffusivity

near 150 K. The ASW diffusivity data provide for an interpretation that does not require either a singularity at 228 K (refs 3, 28, 29) or a new distinct phase of liquid water^{16,17}. The continuity interpretation is supported by additional experimental evidence. The markedly non-Arrhenius behaviour of the VFT fit is consistent with that of a fragile liquid^{5,27} having an ideal glass transition temperature, $T_0 = 118$ K (ref. 4). This value is consistent with the experimental calorimetric glass transition temperature, T_g , observed between 124 and 136 K (refs 30, 31). The rapid decrease in diffusivity on cooling towards the glass temperature is also consistent with the observation of no translational diffusion at 125 K within 13.5 hours (ref. 15). Recent mechanical deformation studies support the liquid-like behaviour of ASW above its T_g (ref. 32). Furthermore, recent low-temperature measurements (163–174 K) indicate that the dielectric relaxation of water has a VFT-like temperature dependence, similar to that shown in Fig. 3 for the self-diffusivity^{32,33}. Further evidence against a low-pressure singularity at 228 K comes from experiments using electron diffraction by large water clusters supercooled to 200 K (ref. 34), and from experiments that measure the velocity distribution of evaporating water molecules from a liquid water jet at 210 K (ref. 35). Additionally, recent computational molecular-dynamics studies of the self diffusion of water molecules in the deeply supercooled liquid state also support the view that no thermodynamic instability is required to explain the anomalous behaviour of the transport properties near the proposed temperature singularity³⁶—although these simulations use the SPC-E water potential which is known to represent inadequately some aspects of the normal liquid behaviour.

Taken together, the above results, combined with our previous demonstration of a thermodynamic continuity¹⁷, suggest that at the calorimetric glass transition temperature of 136 K (ref. 31) the

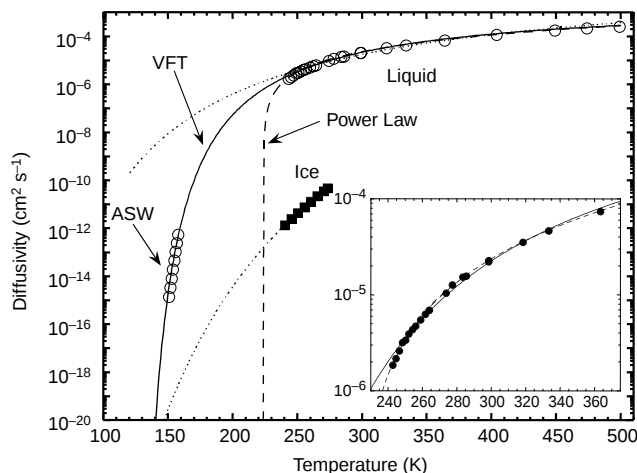


Figure 3 Temperature dependence of diffusivity. Open circles show the ASW diffusivity in the temperature range 150–157 K. Also shown are data for the diffusivity of liquid²⁴ and supercooled liquid^{25,26} water (open circles) and crystalline ice^{22,23} (filled squares). The dotted lines are Arrhenius extrapolations of these diffusivities. The solid line labelled VFT is a fit of the liquid/supercooled liquid data and ASW diffusivity data to the Vogel-Fulcher-Tammann (VFT) equation, $D = D_0 e^{-E/(RT - T_0)}$, where $D_0 = 3.06 \times 10^{-3} \text{ cm}^2 \text{ s}^{-1}$, $E = 892 \text{ K}$ and $T_0 = 118 \text{ K}$ are the parameters. The dashed line labelled Power Law is a fit to the high-temperature ($T > 240 \text{ K}$) liquid/supercooled liquid data to the power-law equation²⁸, $D = D_0 T^{1/2} (T/T_s - 1)^{\gamma}$, where $D_0 = 8.9 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $\gamma = 1.75$ and $T_s = 223.4 \text{ K}$ are the parameters. While the power-law and VFT equations fit the high-temperature data reasonably well, the power law is incompatible with the new low-temperature ASW data reported here. Inset, expanded scale view of the high-temperature diffusivity data^{24–26} (filled circles) and the VFT (solid line) and power-law (dashed line) fits. Above 250 K, both the VFT and power laws give reasonable fits, but below 250 K the VFT curve misses the data by ~25% and the power law fits the data better.

amorphous solid melts into a deeply supercooled metastable extension of normal liquid water before crystallizing near 160 K. This interpretation does not require the existence of a temperature singularity near 228 K (ref. 2) at low pressure ($<0.1 \text{ MPa}$), and is consistent with (but is not proof of) the existence of a second critical point near 220 K and 0.1 GPa (refs 8, 12). Although our ASW diffusivity data provide support for a continuity between ASW and liquid water at low pressure, an unambiguous resolution of the continuity conundrum must await further experiments in the unexplored temperature region from 160 to 240 K. Such experiments will be difficult because of the rapid crystallization of supercooled liquid water below 230 K, and of ASW above 160 K. □

Received 31 December 1998; accepted 17 February 1999.

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Acknowledgements. We thank C. A. Angell, H. E. Stanley, R. J. Speedy, P. Debenedetti, E. Mayer, S. Sastry, P. Poole, F. Sciortino, F. Starr, H. D. Lüdemann and A. Geiger for discussions. This work was supported by the US Department of Energy, Office of Basic Energy Sciences, Chemical Sciences Division. Pacific Northwest National Laboratory is operated for the US Department of Energy by Battelle.

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Structure of the polymer electrolyte poly(ethylene oxide)₆:LiAsF₆

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Polymer electrolytes—salts (such as LiCF₃SO₃) dissolved in solid, high-molar-mass polymers (for example, poly(ethylene oxide), PEO)^{1–3}—hold the key to the development of all-solid-state rechargeable lithium batteries⁴. They also represent an unusual class of coordination compounds in the solid state⁵. Conductivities of up to 10^{–4} S cm^{–1} may be obtained, but higher levels are needed for applications in batteries^{5–7}. To achieve such levels requires a better understanding of the conduction mechanism, and crucial to this is a knowledge of polymer-electrolyte structure. Crystalline forms of polymer electrolytes are obtained at only a few discrete compositions. The structures of 3:1 and 4:1 complexes (denoting the ratio of ether oxygens to cations) have been determined^{5,8,9}. But the 6:1 complex is of greater interest as the conductivity of polymer electrolytes increases significantly on raising the polymer content from 3:1 to 6:1 (refs 10, 11). Furthermore, many highly conducting polymer-electrolyte systems form crystalline 6:1 complexes whereas those with lower conductivities do not. Here we report the structure of the PEO:LiAsF₆ complex with a 6:1 composition. Determination of the structure was carried out *ab initio* by employing a method for flexible molecular structures, involving full profile fitting to the X-ray powder diffraction data by simulated annealing¹². Whereas in the 3:1 complexes the polymer chains form helices, those in the 6:1 complex form double non-helical chains which interlock to form a cylinder. The lithium ions reside inside these cylinders and, in contrast to other complexes, are not coordinated by the anions.

The crystal structure of PEO₃:NaI was solved by fibre diffraction and those of a few similar compounds by powder X-ray diffraction^{8,9,13,14}. The crystal structures of the 1:1 complexes, for example PEO₁:NaSCN and PEO₁:NaCF₃SO₃ have also been established^{15,16}. We have elected to study the structure of the complex PEO₆:LiAsF₆ (ref. 1). In part this is because of the significance of the 6:1 structure to the ionic conductivity of polymer electrolytes in general but also because solving a more dilute crystal structure permits an understanding of how salt content influences structure by comparison with the compositions already investigated.

A preparative method for PEO₆:LiAsF₆ was devised that yielded excellent powder diffraction data (Fig. 1). It involved grinding together, at liquid N₂ temperature, the two dried constituents then loading the mixture, at room temperature in a glove box, into a 0.7-mm-diameter quartz capillary. Once sealed, this was heated at 120 °C for 4 h then annealed at 65 °C for 7 days.

Structure elucidation required the use of a powerful *ab initio* approach based on simulated annealing and developed by us for solving complex structures composed of highly flexible molecules from powder diffraction data¹². For the purpose of solution the asymmetric unit of the unit cell was partitioned into three separate fragments: a Li⁺ cation, a segment of a PEO chain consisting of six ethylene oxide groups and an AsF₆[–] anion. Atomic positions are expressed not as crystallographic coordinates but in terms of internal stereochemical descriptors: bond lengths, bond angles and torsion angles, with respect to an atom chosen as the origin¹². Starting from randomly chosen values we varied 79 parameters including 23 bond lengths, 25 bond angles and 15 torsion angles simultaneously in a Monte Carlo fashion to create trial structural models. Each model was used to calculate a powder pattern which,

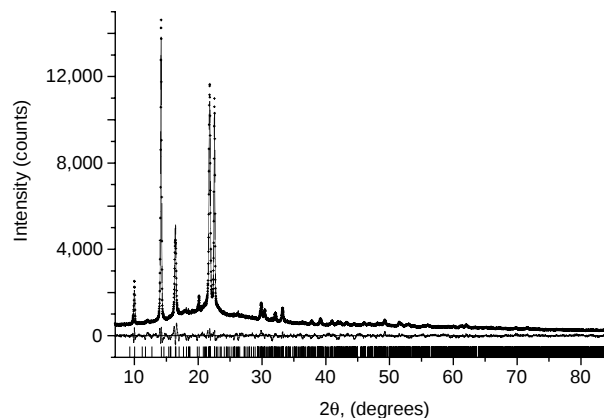


Figure 1 Fitted X-ray powder diffraction pattern of PEO₆:LiAsF₆. Crosses, observed profile; solid lines, calculated (upper) and difference (lower) profiles. Vertical lines represent the positions of reflections. The data were collected on a STOE STADI/P diffractometer operating in transmission mode with CuKα₁ radiation and a small-angle position-sensitive detector. Step width was 0.02°. The pattern was indexed by means of the TREOR²³ program. The unit cell is monoclinic with refined lattice parameters $a = 11.871(4)$ Å, $b = 17.537(3)$ Å, $c = 9.228(3)$ Å, $\beta = 108.21(1)^\circ$. The space group is $P2_1/a$.

in its turn, was compared with the experimental powder diffraction profile (3,650 data points, 1,130 reflections); the agreement factor $\chi^2 = \sum_i (y_i^{\text{obs}} - y_i^{\text{cal}})^2 / y_i^{\text{obs}}$, where y_i is the intensity at the i th point of the pattern, between the two profiles was then calculated. The simulated annealing process located the global minimum in χ^2 producing a structural model with a continuous PEO chain (no assumption of chain continuity was made *a priori*). Given the structural complexity of this polymer electrolyte (79 degrees of freedom) and the absence of a suitable starting structural model, the structure solution represents, to our knowledge, the most complex molecular structure solved *ab initio* from powder diffraction data. The location of the Li⁺ ion was verified by using routine Rietveld refinement of neutron diffraction data¹⁷. The sample for neutron diffraction was prepared with deuterated PEO (Polymer Source). Data were collected on the OSIRIS diffractometer at ISIS, Rutherford Appleton Laboratory. The final structural model (50 atoms in the asymmetric unit) obtained after refinement by means of the GSAS package¹⁸ produced an excellent fit to the diffraction pattern with $\chi^2 = 3.2$ ($R_p = 4.9\%$, $R_{wp} = 6.6\%$, 3,899 data points, 1,370 reflections, 168 variables, 135 soft constraints).

The structure of PEO₆:LiAsF₆ is distinct from all known crystal structures of PEO:salt complexes. The Li⁺ cations are arranged in rows, with each row located inside a cylindrical surface formed by two PEO chains (Fig. 2). Each chain adopts a non-helical conformation that defines a half-cylinder; the two half-cylinders, which are related by a centre of inversion, interlock on both sides. Six ethylene oxide units are required to represent the chain conformation, which, starting from a C–O bond, can be described as *ctggctgcctctgtgctgct*. The conformation was derived by assuming that torsion angles in the range $0 \pm 45^\circ$ are *cis* (*c*), $180 \pm 45^\circ$ are *trans* (*t*), and the rest are either *gauche* (*g*) or *gauche-minus* (*ḡ*). Each cation is coordinated simultaneously by both chains, involving three ether oxygens from one and two from the other, giving a total coordination around Li⁺ of 5 and forming a somewhat distorted square pyramid (see Fig. 2b). Each ether oxygen is coordinated to only one Li⁺ ion and the sixth ether oxygen is not involved in cation coordination. In contrast to other known PEO complexes, the anions do not coordinate the cations but instead are located outside the dimensions of the cylinder in the interchain space. The C–C, C–O and Li–O distances are typical of those found in other PEO complexes, and the As–F distances are within the range observed in other compounds. The Li–Li distances along and between chains are respectively 5.4(1) and 9.2(1) Å.

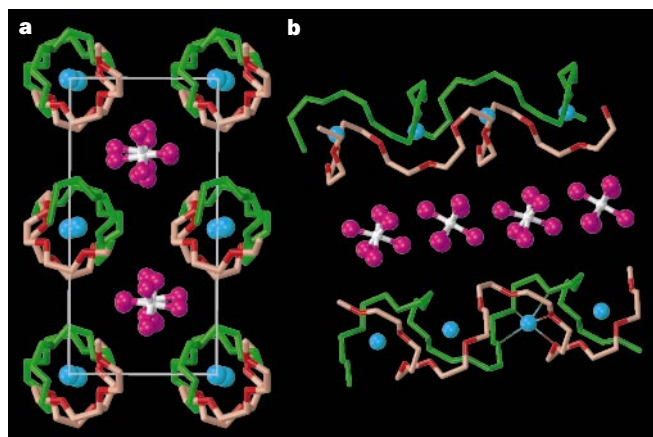


Figure 2. The structure of poly(ethylene oxide)₆:LiAsF₆, hydrogen atoms not shown. **a**, View of the PEO₆:LiAsF₆ structure along *a*, showing rows of Li⁺ ions perpendicular to the page. Blue spheres, lithium; white spheres, arsenic; magenta, fluorine; light green, carbon in chain 1; dark green, oxygen in chain 1; pink, carbon in chain 2; red, oxygen in chain 2. **b**, View of the structure showing the relative positions of the chains and their conformations. Thin lines indicate coordination around the Li⁺ cation. The lithium–ether oxygen distances (Å) for chain 1 are 2.07(5), 2.26(4), 2.28(4); for chain 2 they are 2.05(5), 2.14(6).

The crystal structures of polymer electrolyte complexes provide a significant insight into the structural features present in the amorphous state at the composition of the complex. This has been demonstrated by variable-temperature studies of PEO₃:LiCF₃SO₃ that show that the structure in the crystalline state is retained above the melting point, with only a loss of register between polymer chains¹⁹. As a result, it is especially interesting to consider how the structural features of the crystalline complexes vary with salt content. The 1:1 complexes contain PEO chains in a zigzag conformation. Each cation is coordinated by only two ether oxygens and four anions. The anions bridge between cations that are coordinated to neighbouring chains, which leads to ionic cross-linking extending throughout the solid¹⁶. The 3:1 complexes contain isolated, helical, PEO chains with cations inside each turn of the helices and coordinated by three (Li⁺) or four (Na⁺) ether oxygens and two anions^{5,14}. The 6:1 complex is, we believe, the first to exhibit double PEO chains coordinating the cations. A very interesting model based on a double helix of PEO chains has been proposed previously for complexes such as PEO₄:KSCN²⁰, although later work demonstrated that only a single helix is present⁹. It is noteworthy that despite the adoption of a single helical structure by the polymer chain in pure PEO as well as in the 3:1 and 4:1 complexes, the chain in the intermediate, 6:1 complex studied here possesses a quite different structure. It is evident from the above that as the ratio of ether oxygens to cations is increased from 1:1 there is increasing cation coordination by the ether oxygens at the expense of the anions. Therefore, it is not unreasonable that two polymer chains are necessary to satisfy the coordination requirements of each Li⁺ cation in the 6:1 complex. The absence of any anion coordination of the cations in the 6:1 complex signals that the contact ion pairing present in the 3:1 complexes has disappeared in the 6:1 complex.

It is interesting to consider the implications of the structure for the mechanism of ionic conductivity, particularly in view of the general trend towards significantly higher conductivity on increasing the polymer content from 3:1 to 6:1. As discussed above, the structure of crystalline PEO₃:LiCF₃SO₃ is largely retained in the conducting amorphous state above *T*_g, at and near this composition, with only a loss of long-range order¹⁹. If the same is true for PEO₆:LiAsF₆, a number of consequences can be considered. The absence of ion pairing would mean that the cations and anions are free to migrate independently, leading to an enhancement of ionic conductivity. Furthermore, the anions interact only weakly with

their environment; as a result, high anion mobility is anticipated. As has been pointed out elsewhere, in addition to ensuring local chain mobility (necessary to facilitate ion transport) it is important to organize or align the polymer chains, providing pathways for facile ion transport along and between chains, if ionic conductivity is to be optimized^{5,21,22}. In this context, note that the 6:1 crystal structure possesses highly aligned cylindrical tunnels for Li⁺ ion transport. If the Li⁺ ions remain in the tunnels when in the amorphous state, the tunnels could promote facile Li⁺ ion transport along the chains, involving the sixth ether oxygen, which is not part of the static coordination environment around Li⁺. Further, provided that the tunnels remain highly aligned, this might assist the transfer of the cations from one tunnel to the next, leading to higher ionic conductivity than would exist in a more disordered structure. In the crystal structure the anions are also somewhat constrained in tunnels because neighbouring PEO cylinders are too close to permit the passage of AsF₆⁻ between them. If the memory of the crystal structure is preserved to a high degree, anion transport might occur preferentially in the chain direction.

Generally, cross-linking between chains in polymer electrolytes restricts the local chain motion and hence decreases conductivity¹⁰. The simultaneous coordination of the cations by two PEO chains in the 6:1 complex constitutes extensive cross-linking between polymer electrolyte chains. But unlike, for example, the 1:1 complexes, such cross-linking does not form a three-dimensional network; instead it serves to introduce pairing of the chains. In this case, ion transport along the tunnels could be facilitated by specific and cooperative local modes of the PEO chains that might not be greatly inhibited by the localized cross-linking. The mere presence of cross-linking does not necessarily impose a significant decrease in ionic conductivity in every case; a more detailed analysis is required.

The above suggestions concerning the implications of the 6:1 structure for the mechanism of ionic conduction, as well as the distinctive effect of localized ionic cross-linking, requires further investigation. Studies are already under way to determine the relation between structure and conductivity. It will also be interesting to examine 6:1 complexes based on other salts of strong acids, such as LiN(SO₂CF₃)₂, to see whether they adopt similar structures to that of PEO₆:LiAsF₆. □

Received 17 December 1998; accepted 1 March 1999.

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Acknowledgements. We thank D. Martin and D. Engberg for their help in collection and treatment of the OSIRIS data. P.G.B. thanks the EPSRC and the Leverhulme trust for financial support.

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A nanometre-sized hexahedral coordination capsule assembled from 24 components

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Molecular capsules consist of closed, hollow frameworks within which encapsulated molecules are isolated from interaction with external molecules¹. In this environment, otherwise reactive molecules can be stabilized^{2–5}. Although some molecular capsules have been prepared by conventional synthetic chemistry¹, recent progress in non-covalent synthesis has allowed the creation of capsules held together by hydrogen bonds^{6–9}. Here we report the use of transition-metal-based coordination chemistry^{10–19} to assemble a stable, nanometre-scale capsule from 24 small components: 18 metal ions and six triangular ligands. The capsule is roughly hexahedral and comprises six edge-sharing triangles with two metal ions on each edge. The internal space has a volume of 900 Å³ and is fully closed to all but very small molecules.

The triangle is the simplest unit for the assembly of polyhedra. To construct molecular polyhedra we designed an exo-hexadentate ligand, 1,3,5-tris(3,5-pyrimidyl)benzene (**1** in Fig. 1), as a triangular assembly unit. This ligand is an almost coplanar triangle and is expected to give edge-sharing polyhedra when its aromatic nitrogens are ligated at the *cis*-coordination site of a metal ion, as shown schematically in Fig. 1. A *cis*-protected palladium(II), Pd(NO₃)₂(en) (**2**, en = ethylenediamine), provides a 90° coordination angle, and has been shown to be useful for constructing well-defined discrete structures on complexation with a variety of exopolydentate ligands^{12,20,21}. Because the angles between the planes of tetra-, hexa- and octahedra do not significantly deviate from 90°, we expected the assembly of such a polyhedron on complexation of ligand **1** with Pd(II) unit **2** (Fig. 1).

Of the several possibilities, the assembly of a molecular hexahedron from **1** and **2** was strongly suggested by proton nuclear magnetic resonance (¹H NMR) observations. When ligand **1** was treated with Pd(II) complex **2** (3 equiv.) in D₂O, we observed the predominant formation of a single component whose ¹H NMR spectrum showed seven singlet-like signals in an integral ratio of 2:2:2:2:2:1:1. This observation confirms that, after complexation, the ligand **1** (of C₃ symmetry) is placed in a less-symmetrical (σ₂) environment with only one symmetry axis passing through a 3,5-pyrimidyl (pym) ring and a core benzene ring (Fig. 2). This

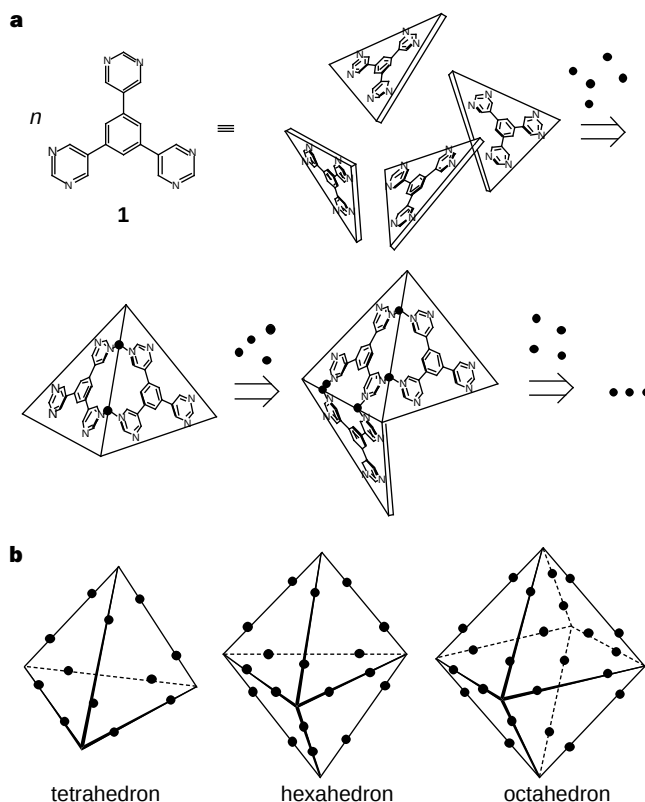


Figure 1 The assembly process of ligand **1** and metal ions (**a**) and the polyhedral frameworks which are expected to assemble (**b**).

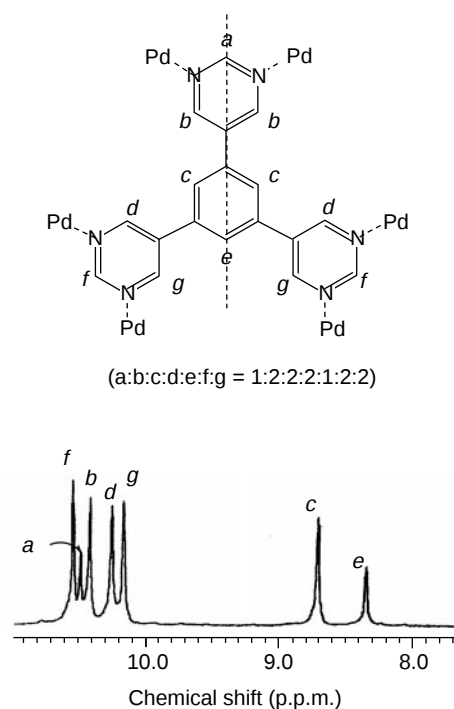


Figure 2 The ¹H NMR spectrum of the product assembling from **1** and **2** (3.3 equiv.). (The spectra were obtained with 500 MHz NMR and show the aromatic region; D₂O was used as the solvent.) The assignments were fully determined by two-dimensional NMR techniques (H–H correlation spectroscopy (COSY), C–H COSY, nuclear Overhauser and exchange spectroscopy (NOESY); and homonuclear Hartman Hahn spectroscopy (HOHAHA), see Supplementary Information).

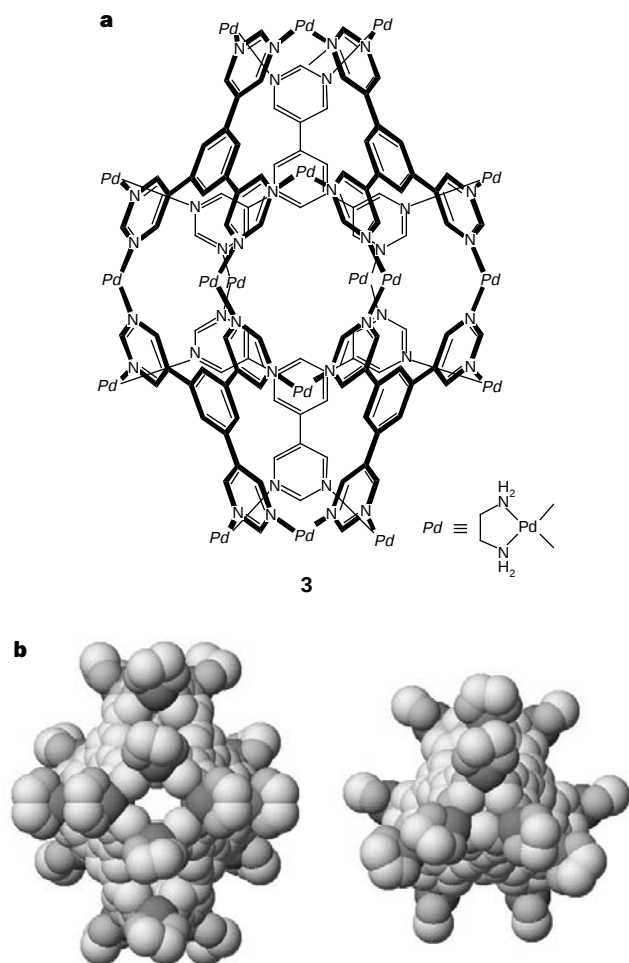


Figure 3 Chemical structure of **3**. **a**, Molecular structure assembled from 18 metal ions (**2**) and six triangular ligands (**1**). **b**, A space-filling model presentation of the X-ray crystal structure of **3** (for details, see Supplementary Information). Left, a view from an equatorial direction; right, a view from an apical direction.

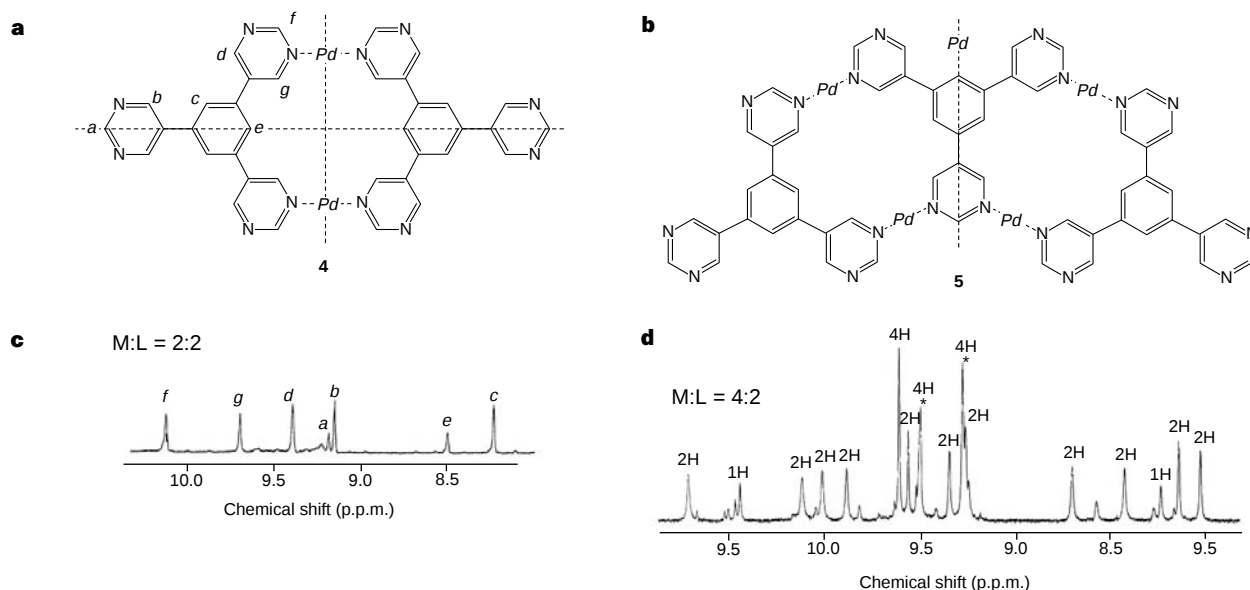


Figure 4 Proposed intermediates in the assembly of **3**. **a**, The structure of dimer **4**. **b**, The structure of trimer **5**. **c**, ^1H NMR (aromatic region) of dimer **4**. The assignments are supported by H-H COSY. **d**, ^1H NMR (aromatic region) of trimer **5**. The integration of signals (36 H in total) assignable to **5** is shown. Two singlet

symmetry is in good agreement with the trigonal bipyramidal structure of molecular hexahedron **3** (see Fig. 3) in which pym groups at the apical corners are non-equivalent to those at the equatorial corners. A small amount of by-products, showing very complex NMR spectra, were also detected. However, the by-product formation was completely suppressed by employing a small excess amount of **2** (3.3 equiv.), suggesting that the by-products and free **2** are in equilibrium with **3**. From the crude reaction solution, complex **3** was isolated in 95% yield as a pure precipitate by adding a large amount of ethanol. Elemental analysis of **3** was consistent with a formula of $3 \cdot (\text{H}_2\text{O})_n$ ($n = 26\text{--}28$).

Reliable evidence for the trigonal bipyramidal structure of hexahedron **3** is provided by X-ray crystallographic analysis. A single crystal of **3** was obtained by slow diffusion of methanol into an aqueous solution of **3** at 4 °C for 4 d, and a diffraction study was done with a CCD diffractometer. The crystal structure of **3** shown in Fig. 3 clearly demonstrates that the assembly is a trigonal bipyramidal coordination capsule with a chemical formula of $\text{C}_{144}\text{H}_{216}\text{N}_{108}\text{Pd}_{18}$, a molecular mass of 7,103 Da, and a dimension of $3 \times 2.5 \times 2.5$ nm (Fig. 3). The pym rings are slightly tilted from the plane of the core benzene ring of ligand **1**. In addition, Pd(II)–pym bonds and pym rings are not completely coplanar. Such small distortions make the pym–Pd–pym coordination angle close to an ideal value of 90° (92–99°). Of 36 nitrate ions involved in **3**, 14 have been found in the crystal structure: of the latter, five are found inside the capsule and nine outside. The other anions are highly disordered and have not yet been located.

Each equatorial corner of the hexahedron is made up by the assembly of four triangle units, where a $(\text{Pd}(\text{II})\text{--pym})_4$ cyclic framework gives a small ‘pinhole’ (2×2 Å). Through these holes, only small molecules such as water and molecular oxygen may pass, but ordinary organic molecules cannot enter or escape. We note that not even such a ‘pinhole’ exists at the apical corners of **3** (Fig. 3b). The free volume inside the capsule, into which guests can be accommodated, is ~ 900 Å³, implying that complex **3** can host large molecules such as buckminsterfullerene, C_{60} .

The thermodynamic stability of **3** is explained by a strong positive cooperative effect of 36 Pd(II)–pym coordination bonds involved in the framework of **3**. Each coordination bond is weak, because coordination by two Pd(II) ions at the 1,3-position of a pym ring

signals with 2H integration may be overlapped, at signals marked by an asterisk. As trimer **5** was observed only at low concentrations, satisfactory ^{13}C NMR and two-dimensional NMR spectra were not obtained.

is unfavourable due to the cationic repulsion between two adjacent Pd(II) ions and the electron-withdrawing effects of adjacent Pd(II)–pym bonds on each other. In fact, macrocycles with (Pd(II)–pym)_n frameworks (*n* = 3, 4) did not assemble from **2** and 1,3-pyrimidine; instead, oligomers showing broad peaks in NMR were observed. A strong positive cooperative effect of Pd(II)–pym bonds should therefore work in the quantitative assembly of a nanosized capsule structure.

The metal-linked dimer **4** and trimer **5** (Fig. 4a, b) may be involved as intermediates in the assembly process of **3**, because these species were observed when ligand **1** was titrated with Pd(II) complex **2** in D₂O. At 1 : 2 = 1 : 1 stoichiometry, NMR displayed the high-yield formation of a single product which was assigned to dimer **4** (Fig. 4c). Dimer **4** was isolated as a salt with PF₆ (96% yield) and characterized by NMR, electrospray mass spectrometry (ESI-MS), and elemental analysis. Further addition of **2** into the D₂O solution made the spectrum very complex. However, the spectrum again became simpler at 1 : 2 = 3 : 4 stoichiometry showing 16 singlet signals with a total integral ratio of 36 H (4H × 3, 2H × 11, 1H × 2) in the aromatic region, consistent with the trimer structure of **5** (Fig. 4d).

The present results complement our previous synthesis of a corner-sharing octahedron assembling from an exo-tridentate ligand, 1,3,5-tris(4-pyridyl)triazine, and Pd(II) complex **2** (refs 12,15,16). The framework of this octahedron complex has large windows and encloses large guest molecules (for example, as many as four carborane molecules) which can enter or exit through the openings. In contrast, the present edge-sharing hexahedron complex **3** has a closed shell structure, and so should be able to encage large molecules. The encapsulation of large guest molecules by this capsule, as well as guest-templated synthesis of other polyhedra (for example, a regular tetrahedron and an octahedron) are now being studied. □

Methods

Synthesis and physical properties of 3. Ligand **1** (31 mg, 0.10 mmol) was suspended in an aqueous solution (3 ml) of **2** (96 mg, 0.33 mmol) and the mixture was stirred at ambient temperature for 24 h. Addition of ethanol (10 ml) to the solution precipitated **3** as pale yellow crystals (120 mg, 95%); melting point (m.p.) ~220 °C (decomposed); ¹H NMR (500 MHz, D₂O tetramethylsilane as external standard) δ 2.9–3.3 (m, 72 H), 8.36 (s, 6 H), 8.73 (s, 12 H), 10.18 (s, 12 H), 10.26 (s, 12 H), 10.43 (s, 12 H), 10.50 (s, 6 H), 10.55 (s, 12 H); ¹³C NMR (125 MHz, D₂O) δ 47.5 (CH₂), 47.6 (CH₂), 47.8 (CH₂), 125.6 (CH), 127.0 (CH), 130.3 (quaternary carbon, Cq), 131.7 (Cq), 133.4 (Cq), 134.7 (Cq), 157.7 (CH), 158.8 (CH), 159.3 (CH), 159.5 (CH), 160.3 (CH); IR (KBr) 3,209, 1,384, 1,057, 879, 709 cm⁻¹. Elemental analysis: calculated for (C₁₄H₂₁N₁₀O₁₈Pd₁₈·27H₂O, C, 22.77; H, 3.56; N, 19.92; found: C, 23.06; H, 3.51; N, 19.60.

Synthesis and physical properties of 4. Ligand **1** (12.5 mg, 0.04 mmol) was suspended in dimethyl sulphoxide (1.0 ml) of **2** (11.6 mg, 0.04 mmol) and the mixture was stirred for 8.5 h at ambient temperature. The pale yellow solution was added dropwise to a saturated aqueous solution (10 ml) of KPF₆ and the resulting white precipitates were filtered to give **4** (PF₆ salts) (31.4 mg, 0.019 mmol, 96%); m.p. ~250 °C (decomposed); ¹H NMR (500 MHz, D₂O) δ 2.76 (s, 8 H), 8.25 (s, 4 H), 8.49 (s, 2 H), 9.17 (s, 4 H), 9.21 (s, 2 H), 9.41 (d, *J* = 2.5 Hz, 4 H), 9.71 (s, 4 H), 10.10 (d, *J* = 2.5 Hz, 4 H); IR (KBr) 1,559, 1,421, 1,399, 1,058, 839, 709, 559 cm⁻¹. ESI-MS *m/z* 1,392.2 (M–PF₆), 1,430.9 (M–PF₆ + CH₃CN), 1,474.0 (M–PF₆ + 2CH₃CN), 1,557.1 (M–PF₆ + 4CH₃CN). Elemental analysis: calcd. for (C₁₈H₁₂N₆)₂[C₂H₈N₂Pd(PF₆)₂·5H₂O, C, 29.52; H, 3.10; N, 13.77; found: C, 29.19; H, 2.76; N, 13.50.

X-ray structural analysis of 3. Ligand **1** (12.5 mg, 0.040 mmol) was suspended in a D₂O solution (1 ml) of **2** (34.9 mg, 0.120 mmol), and methanol (14 μl) was added to the mixture. After stirring at ambient temperature for 17 h, diffusion of methanol into the resulting pale yellow solution at 4 °C for 4 d gave yellow single crystals of **3**. A single crystal of **3** (0.25 × 0.30 × 0.35 mm) was mounted on a glass fibre. All measurements were made on a charge coupled device (CCD) plate area detector with graphite monochromated Mo–Kα radiation. The data were collected at 296 K. Crystal data for **3**: formula C₁₄₄H₂₁₆N₁₀₈O₁₀₈Pd₁₈·27H₂O, *M* = 7,589.56, hexagonal, space group *P*6₃/*m* (#176), *a* = 23.168(8), *c* = 34.10(2) Å, *V* = 15851(16) Å³, ρ_{calcd.} = 1.60 g cm⁻³, *Z* = 2, *F*(000) = 7,596,

μ(MoKα) = 11.03 cm⁻¹, λ(MoKα) = 0.71069 Å; 99,000 reflections measured, 3,034 observed (*I* > 3.50σ(*I*)); number of variables 425; *R*₁ = 0.129; *wR*₂ = 0.166.

Received 16 October 1998; accepted 23 February 1999.

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

Acknowledgements. We thank K. Biradha for part of the crystallographic studies.

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Self-assembly of nanoscale cuboctahedra by coordination chemistry

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Self-assembled polyhedral structures are common in biology. The coats of many viruses, for example, have a structure based on icosahedral symmetry¹. The preparation of synthetic polyhedral molecular assemblies represents a challenging problem, but supramolecular chemistry^{2–4} has now advanced to the point where the task may be addressed. Macromolecular and supramolecular entities of predefined geometric shape and with well-defined internal environments are potentially important for inclusion phenomena^{5–8}, molecular recognition^{5,6} and catalysis⁹. Here we report the use of self-assembly of molecular units driven by coordination to transition-metal ions¹⁰ to prepare a cubocta-

hedron from 20 tridentate and bidentate subunits in a single step. The cuboctahedron is an archimedean semiregular polyhedron that combines square and triangular faces. Our self-assembled polyhedral capsules, characterized by NMR and electrospray mass spectrometry, are around 5 nanometres in diameter.

Figure 1 illustrates our design approach. The shape of a cuboctahedron can be achieved by combining planar tridentate faces with 108° turning angles via bidentate angular components. As 109.5° is close to 108° , the bidentate angular linking subunit may have a tetrahedral atom connected to a rigid linear donor or acceptor site. The tridentate building unit must, in turn, have three donor or acceptor sites located in one plane at 120° relative to each other. The ratio of bidentate to tridentate ligands must be 3:2 with a total of 20 appropriate subunits in order to obtain the closed structure. It is also very important for the resulting assembly to be soluble in the reaction medium and most importantly, the product must be thermodynamically stable but kinetically labile, so any errors in the assembly can be self-corrected.

The tridentate unit, 1,3,5-tris(4'-iodophenyl)ethynylbenzene was prepared from 1,3,5-triethynylbenzene via cross-coupling; the triple oxidative addition of $\text{Pt}(\text{PPh}_3)_4$ into the iodo-phenyl bond was carried out at 95°C in toluene, then halide abstraction with AgOTf in CD_2Cl_2 afforded the desired precursor **1** (Fig. 1; OTf indicates triflate, OSO_2CF_3). The bidentate angular donor unit with the required tetrahedral carbon, 4,4'-bispyridylacetal (**3** in Fig. 2), was prepared from the known 4,4'-bispyridylketone (ref. 11).

The self-assembly was carried out as summarized in Fig. 2. Slow addition of a dichloromethane solution of **3** at 25°C to a solution of the transition-metal tris(triflate) in the same solvent resulted in a single highly symmetrical entity with a stoichiometry of 3:2, as determined by integration of the relevant signals in the ^1H NMR. The ^{31}P -NMR spectrum of cuboctahedron **5** exhibited only a singlet along with the ^{195}Pt satellites. The product is a robust, hygroscopic microcrystalline solid with a relatively high-temperature decomposition point. This molecule, despite its large molecular mass (26,592.2 Da) and high charge (24+) is remarkably soluble in organic solvents, such as dichloromethane or acetone.

A second cuboctahedron was assembled via a different route, where the tridentate angular component 1,3,5-tris(4-pyridylethynyl)benzene (**2** in Fig. 1) contained the donor sites, while the bidentate unit, bis(4-[*trans*- $\text{Pt}(\text{PPh}_3)_3\text{OTf}$]phenyl)ketone **4**¹¹ was selected as the acceptor. The ketone carbon is trigonal, with a 120° bond angle, but if we account for the large size of the resulting assembly, this 12° distortion can be accommodated by a slight distortion within its subunits. The preparation of **6** was also performed by mixing a solution of the angular ligand **2** with a solution of subunit **4** in dichloromethane (Fig. 2). The ^1H and ^{31}P NMR spectra again indicated the formation of a single, highly symmetrical entity. The multinuclear NMR, infrared, elemental analyses and physical properties (solubility, high melting point) of **5** and **6** all are consistent with the design-based cuboctahedral structures. A molecular model of **6** obtained from force-field

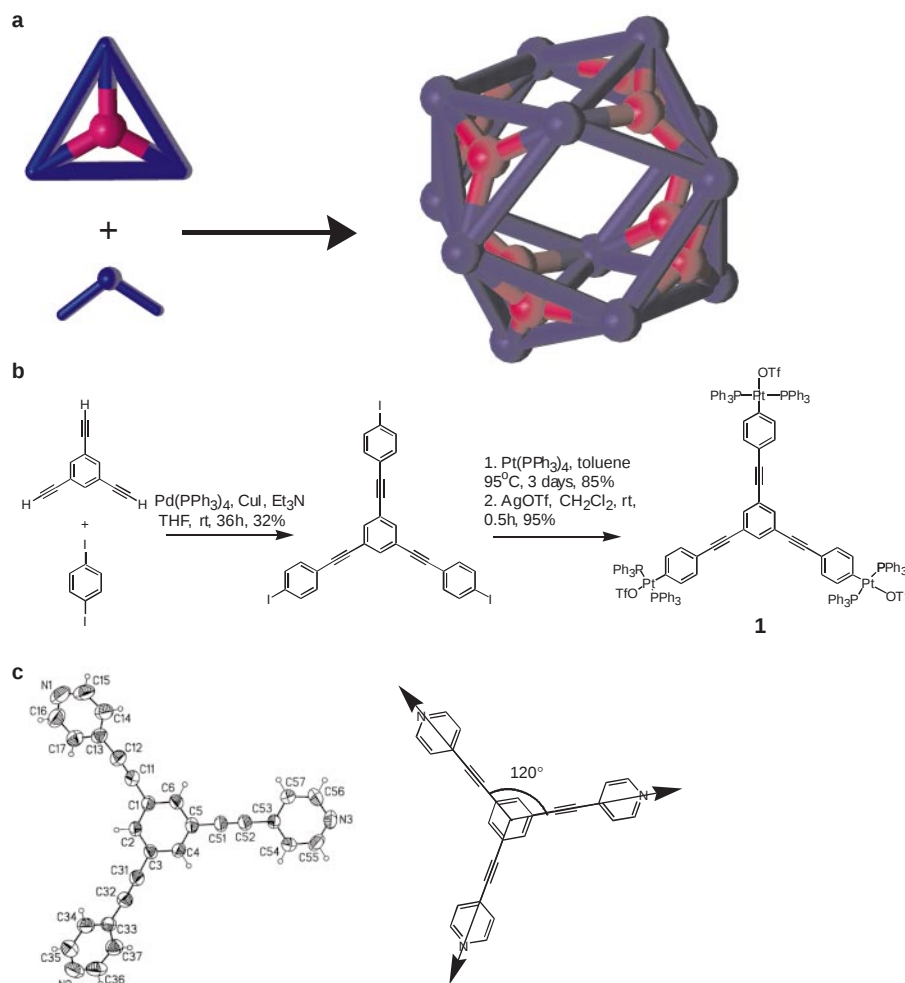


Figure 1 The general design scheme for the cuboctahedron. **a**, Assembly from a combination of bidentate linear and tridentate angular components. 1,3,5-tris(4'-

iodophenyl)ethynylbenzene (**b**) and 1,3,5-tri-(4-pyridyl)ethynylbenzene (**c**) exemplify tridentate angular components with 120° directing angles.

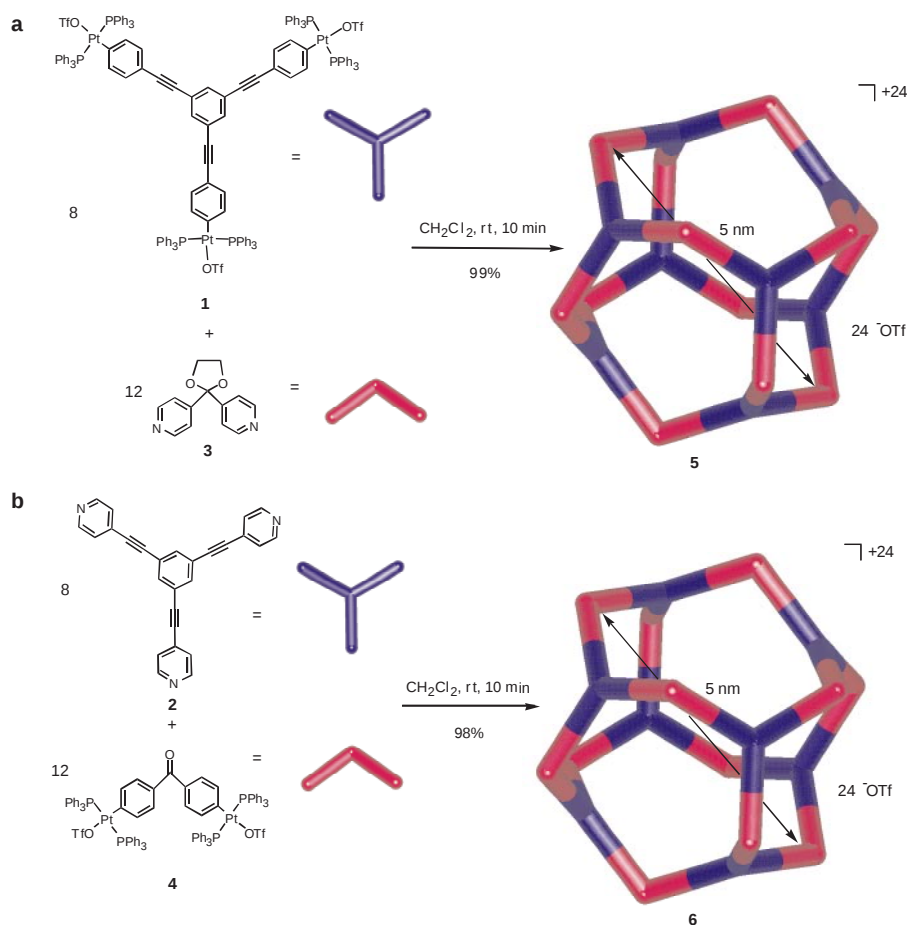


Figure 2 The reaction schemes for the preparation of nanoscopic cuboctahedra **5(a)** and **6(b)**.

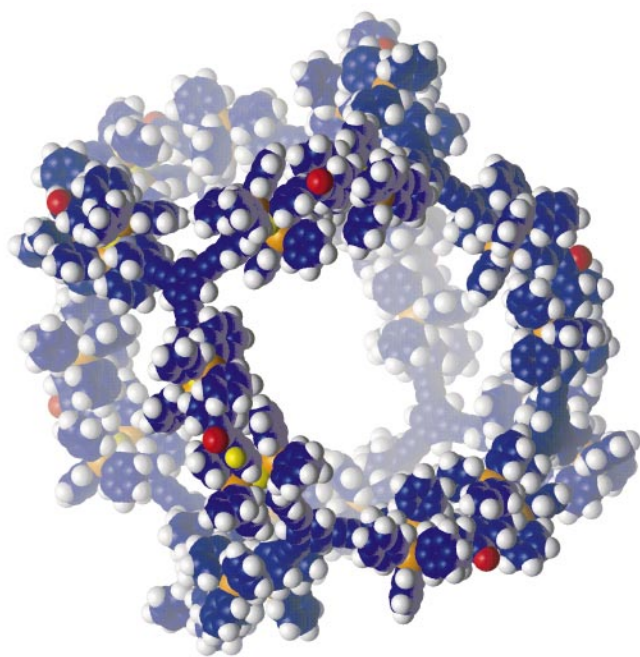


Figure 3 Space-filling model of the cuboctahedron **6** derived from ESFF (Extensible Systematic Force Field) simulations using the Insight II 97.0 software package¹⁵.

simulations¹² is presented in Fig. 3. We believe that these macro-molecules are the first members of a unique class of artificial synthetic assemblies of O-symmetry.

The size of **6** was assessed experimentally by measuring its self-diffusion coefficient using the pulsed gradient spin echo (PGSE) NMR technique¹³, widely employed to study aggregation and diffusion data of various chemical and biological assemblies in solution. The self-diffusion coefficient¹⁴ for **6** at 25 °C is $(1.92 \pm 0.072) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ which gives an experimental hydrodynamic diameter of 5.0 nm, in agreement with predictions based on the force-field simulations as well as the size of the component building units.

Electrospray ionization mass spectrometry (ESI-MS) was used to identify the respective compositions of **5** and **6**. On gentle ionization, the 12+, 13+, 16+ and 18+ species with mass-to-charge ratios (m/z) of 2,067, 1,896, 1,513 and 1,329, respectively, were observed for **5**, resulting from the loss of the appropriate number of triflate counterions. Fragmentation of **5** was also observed to a lesser degree, with the main components consisting of several combinations of precursors **1** and **3** with the loss of triflate counterions. Identification of fragments aided in determining coincidental combinations. For example, the peak found at 2,067 m/z was identified as the fragment combination of $(2) \times (1) + (3) \times (3)$ with a 3+ charge state resulting from the loss of three triflate counter ions, not simply **5** without 12 triflates (12+ charge state). The composition of **6** was supported by the observation of the 10+, 11+, 12+, 14+, 15+ and 16+ species resulting from the loss of triflate counter ions (m/z 2,456, 2,219, 2,023, 1,713, 1,588 and 1,480, respectively). Additional evidence for **6** was given by the careful

analysis of the fragmentation data containing expected combinations of precursors **3** and **4**.

The coordination-driven self-assembly process described here facilitates the ready formation of single-molecule, highly symmetrical, static, non-dissociating ultrafine particles by simple mixing of predesigned, readily available components. This provides rapid access to supramolecular ensembles that are much larger than any classical synthetic macrocycles known at present. □

Methods

Preparation. A 5-ml round-bottom flask in a glovebox was charged either with 1,3,5-tris[4-(*trans*-Pt(PPh₃)₂OTf) phenylethynyl] benzene **1** (30 mg, 0.01 mmol) for cuboctahedron **5** or with bis[4-(*trans*-Pt(PPh₃)₃I)phenyl] ketone **2** (30 mg, 0.007 mmol) in 2 ml of CD₂Cl₂. Then, a solution of 4,4'-bispyridylacetal **3** (3.5 mg, 0.015 mmol) in 2 ml of CD₂Cl₂ was added dropwise to the solution of **1** or 1,3,5-tris(4'-pyridylethynyl)benzene **4** (3.2 mg, 0.011 mmol) was added to **2**, resulting in the solutions of cuboctahedrons **5** or **6**, respectively. The products were isolated by solvent removal *in vacuo*. Yields: 33.5 mg (99%) for **5** or 32 mg (98%) for **6**.

Characterization. The identity and purity of the products of self-assembly and their precursors was confirmed by ¹H, ³¹P{¹H}, ¹³C{¹H} and ¹⁹F NMR, IR, ESI-MS and elemental analyses: see Supplementary Information.

PGSE technique. A Stejskal-Tanner pulse sequence was used¹³. The diffusion coefficients for both **5** and **6** were determined using ³¹P resonances in dichloromethane-acetone mixtures at 25°C on the Z-gradient probe capable of producing pulsed field gradients of nearly 200 G cm⁻¹. The molecular size was determined from the effective hydrodynamic radius *R*, related to the effective hydrodynamic volume *V_m* as *V_m* = 4(π/3)*R*³. *R* is calculated from the Stokes-Einstein equation, *D* = (*k_B**T*)/(6π*R*η), where *D* is the diffusion coefficient, *k_B* is the Boltzmann constant, *T* is the absolute temperature, and η is the viscosity of the liquid through which the species is diffusing¹⁴.

ESI-MS technique. Samples of **5** and **6** were dissolved in acetone (10–20 ng μL⁻¹) and injected via syringe pump (100-μL syringe) into a Micromass Quattro II mass spectrometer with ionization performed under electrospray conditions (flow rate, 7.7 μL min⁻¹; capillary voltage, 3.0 kV; cone, 47 V; extractor, 27 V). About 15 individual scans were averaged for the mass spectrum. The calibration of the mass range 500–3,000 atomic mass units was done with a 1:1 mixture of an isopropanol:water solution of NaI (2 μg μL⁻¹) and CsI (0.01 μg μL⁻¹). A table of ESI-MS data for **5** and **6** is available; see Supplementary Information.

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Supplementary information is available on Nature's World - Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

Acknowledgements. This work was supported by the National Science Foundation and the National Institutes of Health.

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Signature of recent climate change in frequencies of natural atmospheric circulation regimes

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A crucial question in the global-warming debate concerns the extent to which recent climate change is caused by anthropogenic forcing or is a manifestation of natural climate variability¹. It is commonly thought that the climate response to anthropogenic forcing should be distinct from the patterns of natural climate variability. But, on the basis of studies of nonlinear chaotic models with preferred states or 'regimes', it has been argued^{2,3} that the spatial patterns of the response to anthropogenic forcing may in fact project principally onto modes of natural climate variability. Here we use atmospheric circulation data from the Northern Hemisphere to show that recent climate change can be interpreted in terms of changes in the frequency of occurrence of natural atmospheric circulation regimes. We conclude that recent Northern Hemisphere warming may be more directly related to the thermal structure of these circulation regimes than to any anthropogenic forcing pattern itself. Conversely, the fact that observed climate change projects onto natural patterns cannot be used as evidence of no anthropogenic effect on climate. These results may help explain possible differences between trends in surface temperature and satellite-based temperature in the free atmosphere^{4–6}.

Our climate can be thought of as a nonlinear dynamical system with a chaotic attractor. Let us imagine that the anthropogenic influence on climate (for example, from increased atmospheric concentration of CO₂) can be represented by a weak imposed forcing on this dynamical system. Because of the nonlinearity of the underlying system, its sensitivity to this forcing will vary with location on the attractor; in regions of strong local instability⁷ the system may be sensitive to the imposed forcing, but in regions of stability the system will be relatively insensitive. This notion can be demonstrated on the chaotic Lorenz⁸ system which has two dominant regimes. Irregular fluctuations between these regimes characterize the model's principal mode of internal variability. Largely independent of the details of the imposed forcing, the response of the Lorenz system to an imposed forcing is associated with an increase in the probability density function (PDF) associated with one regime, and a decrease in the PDF associated with the other regime (Fig. 1). (In the unforced Lorenz model, the PDF has the same value at both centroids.) By contrast, the locations of the regimes' centroids in phase space are largely unaffected by the imposed forcing. This can be understood by noting that the centroids of the regimes are phase-space regions of relative stability; the system is sensitive to the imposed forcing in a region of the attractor between the two centroids.

If this picture were applicable to the real climate system, it would imply that anthropogenically forced changes in climate would project primarily onto the principal patterns of natural variability, even though such natural variability may occur predominantly on

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timescales much shorter than that of the imposed forcing.

To validate this approach, we performed a study on monthly-mean objective analyses of Northern Hemisphere (NH) extended-winter (November to April) 500-hPa geopotential height—essentially the height of a constant pressure surface in the mid-troposphere—from the US National Centers for Environmental Prediction (NCEP), from 1949 to 1994, on a regular latitude/longitude grid with 2.5-degree spacing. First, the seasonal cycle has been removed from the data by computing anomalies with respect to the long-term monthly mean. Second, the data is further detrended by taking deviations from a 5-year running mean. An empirical orthogonal function (EOF) analysis is then applied to this detrended data in order to define a reduced phase space based on patterns of variability with timescales shorter than the observed decadal trends. The resulting EOFs are very similar to those shown in ref. 9, and are not shown here for brevity. (The structure of the EOFs can also be deduced from the PDF maxima shown below.) Whilst the first two EOFs only explain 27% of monthly mean variance (32% of 5-year mean variance), as discussed below, their significance lies in the fact that their spatial structure is well correlated with recent climate-change patterns.

Each panel in Fig. 2 shows a PDF of the projection coefficients of the non-detrended monthly mean height field, in the reduced phase space spanned by the first two detrended EOFs. These PDFs have been generated using a gaussian-kernel estimator¹⁰, with a smoothing parameter large enough to detect multimodality with statistical significance. Figure 2a shows the PDF for the whole period (1949–94). There are four maxima, labelled A, B, C, D in Fig. 2a. Consistent with earlier studies^{9,11,12}, the presence of these maxima confirms that the PDF of the climate attractor is multimodal, and therefore cannot be described adequately using a multinormal distribution. The geographical patterns of these density maxima are illustrated in Fig. 3, where they are shown as departures from the 1949–94 mean 500-hPa height.

Cluster A (Fig. 3) is a regime whose spatial structure is very similar to the pattern found by linear regression analysis between monthly-mean NH-mean surface air temperature and 500-hPa height (compare cluster A with Figure 5 in ref. 13). Essentially, this pattern denotes the manifestation in 500-hPa geopotential height of the “cold ocean warm land” (COWL) pattern, which, to a first approximation, describes much of the recent climate change of NH surface air temperature^{1,13}. Hence, although cluster A is

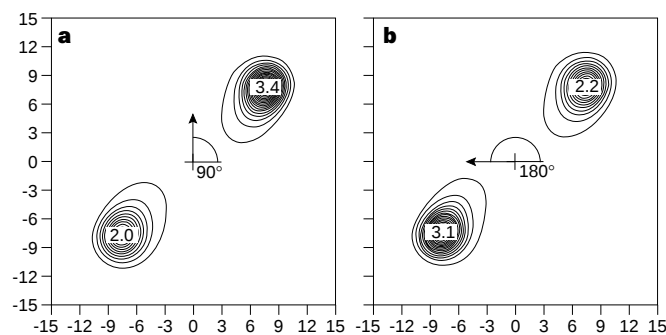


Figure 1 Response of a nonlinear chaotic model to imposed forcing. Illustrated is the state vector PDF (invariant measure) of a forced Lorenz³ attractor, with governing equations $\dot{X} = -10X + 10Y + 2.5 \cos \theta$, $\dot{Y} = -XZ + 28X - Y + 2.5 \sin \theta$, $\dot{Z} = XY - (8/3)Z$, in X, Y phase space. The arrow shown is the forcing vector ($2.5 \cos \theta, 2.5 \sin \theta$) in this two-dimensional phase space. A short running time-average has been applied to the state vector to emphasise the regime character of the Lorenz attractor. **a**, $\theta = 90^\circ$; **b**, $\theta = 180^\circ$. The numbers shown correspond to maxima of the PDF at the regime centroids. The quantities plotted on the horizontal and vertical axes are values of X and Y respectively. See ref. 3 for details.

defined from intraseasonal and interannual variations, a NH-mean surface temperature warming trend would be consistent, in the nonlinear approach, with an increase in residence frequency of cluster A. The height anomalies associated with clusters B and C (Fig. 3) have projection onto the negative Pacific North American¹⁴ (PNA) pattern. The existence of multiple regimes with negative PNA index is consistent with studies^{11,15} showing relatively high intraseasonal variability during periods of negative PNA-index circulation. Cluster B has a projection onto the positive North Atlantic Oscillation¹⁶ patterns, whilst cluster D (Fig. 3) shows a pattern with most amplitude over the North Atlantic, extending over the Arctic. In fact, cluster D is extremely well correlated with the 500-hPa height component of the so-called Arctic Oscillation¹⁷ in its negative phase.

The nonlinear picture outlined above applies to anomalous forcing from the ocean, as well as to anthropogenic forcing. Hence, in one test of the approach, we recomputed the two-dimensional PDF from the NCEP data, with all El Niño/La Niña years removed (using the stratification proposed in ref. 18). This results in 13 warm El Niño years and 7 cold La Niña years being removed from the data: results are shown in Fig. 2b. It can be seen that the four clusters of Fig. 2a continue to be observed in the reduced data set; in other words, none of the regimes A–D owes its existence to forcing by El Niño. However, El Niño does affect the regime frequencies; most noticeably, the frequency of occurrence of cluster A (associated with the COWL pattern) is reduced. The ability of El Niño to excite a response pattern similar to cluster A has been noted elsewhere^{3,19}. The reduction in the PDF of cluster A is

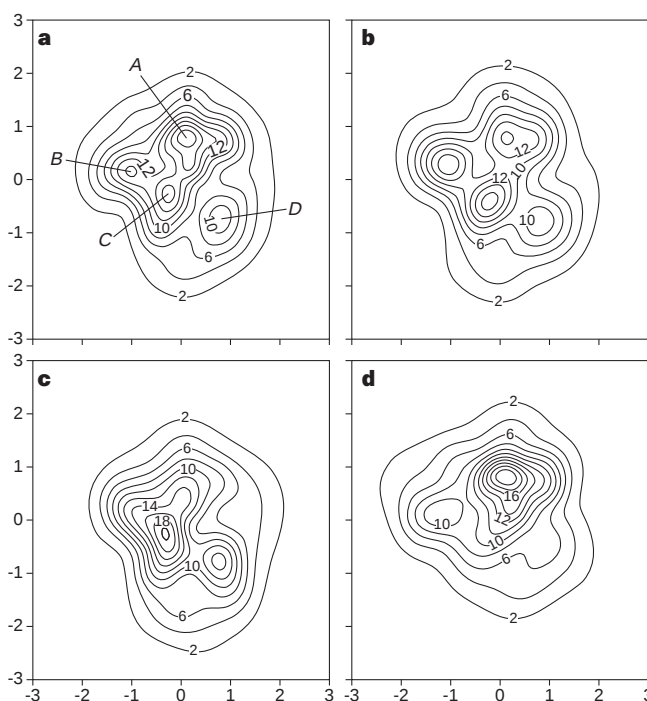


Figure 2 Evidence of recent change in the climate attractor. Illustrated is the atmospheric state vector PDF based on monthly-mean 500-hPa geopotential height in the space spanned by the two dominant atmospheric EOFs computed from detrended monthly mean timeseries. See text for details. **a**, Using all data from the 1949–94 period. See text for details about maxima A, B, C, D. **b**, Using data from the 1949–94 period, from which all months in which El Niño/La Niña events occurred, have been removed. **c**, Using data from the 1949–71 sub-period. **d**, Using data from the 1971–94 sub-period. The quantities plotted on the horizontal and vertical axes are projection coefficients of geopotential height onto the first and second EOFs respectively. We note that the same detrended EOFs are used in all four panels, hence the origin refers to the same atmospheric state in each panel.

consistent with the removal of more warm El Niño events than cold La Niña events from the full data set.

We now return to the changes in the PDF on decadal timescales. PDFs for the sub-periods 1949–71 and 1971–94 are shown in Fig. 2c and d, respectively. It can be seen that in the first-half period, the PDF associated with cluster A is reduced (compared with long-term values), whilst the PDFs associated with clusters C and D are enhanced. In the second-half period, on the other hand, the PDF associated with cluster A is strongly enhanced, whilst the PDFs associated with all the other clusters are reduced. Comparing Fig. 2c and d with Fig. 2a, it can be seen that the phase-space location of the regimes is relatively stable despite these large changes in the PDF.

The decrease in the PDF of cluster D in the second-half period is consistent with what has been noted¹⁷ as a “systematic bias in one of the atmosphere’s dominant naturally-occurring modes of variability”. On the basis of the approach presented here, it is possible that this bias is a response to anthropogenic forcing (recent model integrations confirm a strong effect of anthropogenic forcing on the Arctic Oscillation²⁵).

The changes in the PDF of the climate attractor are consistent with more conventional analyses of recent climate change. For example, in Fig. 4 we show the time series of the projection coefficients of the non-detrended height anomalies onto the first and second detrended EOFs. Both seasonal and 10-year running mean filters have been applied to the time series of the monthly-mean coefficients. The time series from the second EOF shows a clear upward trend, consistent with the increase in the frequency of cluster A (which, from its position in the reduced phase space of Fig. 2, can be described almost exactly in terms of the second EOF pattern). In fact, much of the recent climate-change signal can be explained in terms of this detrended EOF. If Δ denotes the difference field between the 1949–71 time-mean geopotential height and the 1971–94 time-mean geopotential height, and $P_2(\Delta)$ denotes the projection of Δ onto the second detrended

EOF, then the spatial correlation between Δ and $P_2(\Delta)$ is 0.6. In contrast, there is no clear trend in the timeseries from the first EOF; however, the evidence of decreased values towards the end of the period is consistent with a decrease in the frequency of occurrence of cluster D (and a corresponding increase in the Arctic Oscillation index).

Overall, our results indicate that in the NH, much of the recent tropospheric climate change can be understood in terms of a change in the frequency of residence of dominant, naturally occurring regimes of NH atmospheric intraseasonal-interannual variability, consistent with the nonlinear approach outlined above. Although the regime structure of the real climate attractor is certainly more complex than the two-regime Lorenz attractor, observed climate change is qualitatively consistent with the simple picture outlined in Fig. 1.

This study has a number of implications. First, we note that just because the geographical distribution of observed climate change correlates well with patterns of natural variability, this cannot be taken to imply an absence of anthropogenic forcing.

Second, the increase in hemispheric-mean temperature in recent years is, in part at least, directly associated with an increase in the frequency of the cluster-A circulation regime. From this point of view, to attribute the observed hemispheric mean surface warming to a direct forcing by the “greenhouse effect” is an oversimplification. If this regime-induced climate change is anthropogenic, then a relevant question is: why should increased CO₂ lead to an increase in the frequency of cluster A? To answer it, one should take into account the fact that large-scale atmospheric patterns are much

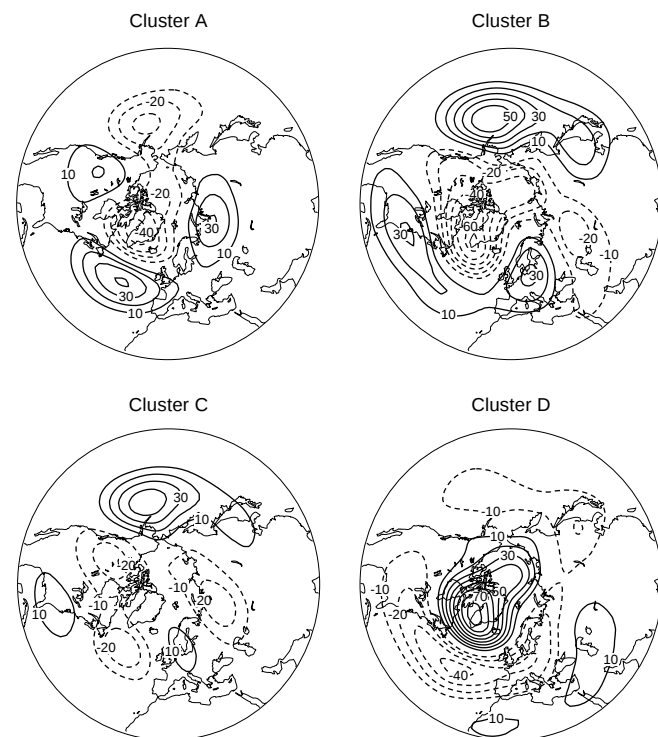


Figure 3 Geographical patterns of the four atmospheric regimes. Shown is the geographical distribution of 500-hPa geopotential height anomaly associated with clusters A (The “cold ocean warm land” regime) B, C and D (the “Arctic Oscillation” regime). Contour interval, 10 m.

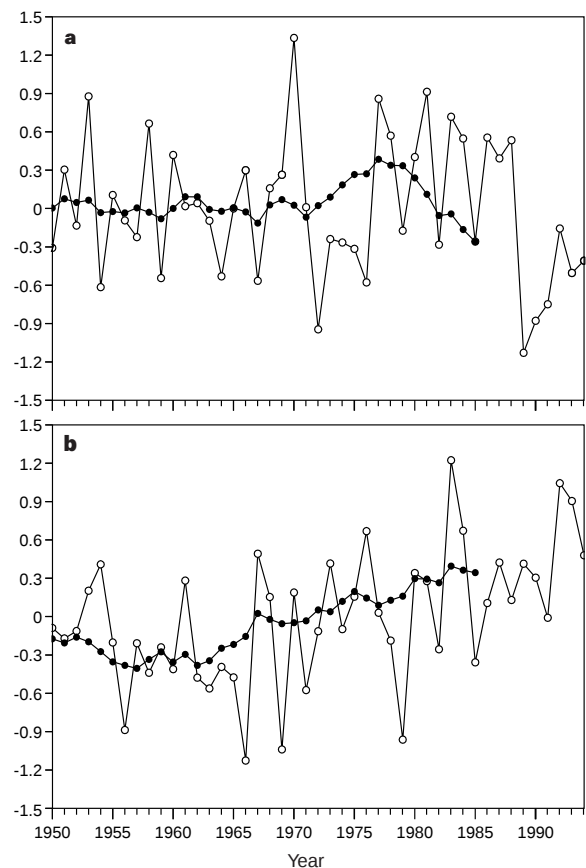


Figure 4 Evidence of climate trends. Shown are time series (1949–94) of the projection coefficients of non-detrended objective analyses of 500-hPa geopotential height onto the two dominant detrended EOFs of 500-hPa geopotential height. Open circles, seasonal averages; filled circles, decadal averages. **a**, Projection coefficient of first EOF. **b**, Projection coefficient of second EOF.

more sensitive to some forcing perturbations than to others. Moreover, because of the non-normality of the relevant dynamical operators^{20–22}, changes in regime frequency will be sensitive to forcing perturbations which may have little spatial correlation with the regimes themselves. Different approaches, all based on adjoint dynamical methods, have been proposed^{20–22} to investigate the structure of these sensitive patterns and their ‘optimal’ forcing fields, either in a stationary or in a time-dependent framework. At present, we do not have a satisfactory understanding of these regime instabilities, and how anomalous radiative forcing projects on the sensitivity patterns.

Third, if the horizontal structure of recent climate change is correlated with the horizontal structure of regimes of natural variability, then, by implication, the same should be true of the vertical structure: specifically, the vertical temperature profile of observed climate change should reflect the vertical temperature profile of the regimes, rather than the vertical structure of any perturbed radiative forcing. Away from the surface layer, temperature anomalies associated with cluster A will be similar in magnitude over sea and over land. However, at the surface, temperature anomalies associated with cluster A will be larger over the land than over the sea, because of the smaller heat capacity of the land surface. Hence the hemispheric-mean temperature anomaly associated with cluster A will be weaker in the free atmosphere than in the surface layer. The signal, however strong or weak it is, should reverse at the level of maximum geopotential anomaly, that is, at about the 300-hPa level. This is consistent with satellite observations in the free atmosphere^{4,5} in spite of the implications of orbital decay⁶. A more complete resolution of this question requires an analysis of the three-dimensional structure of these circulation regimes; the so-called reanalysis datasets²³ would be well-suited for this task.

Last we note that these results indicate that predictions of anthropogenic climate change require models which can simulate accurately natural circulation regimes and their associated variability, even though the dominant timescale of such variability may be much shorter than the climate-change signal itself. More generally, these models should be able to simulate the non-gaussian characteristics of the climate attractor. As yet, few tests have been performed on climate models to assess this component of their behaviour²⁴. □

Received 14 December 1998; accepted 10 March 1999.

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Acknowledgements. S.C. and F.M. were supported by the Commission of the European Communities under project MILLENNIA (Numerical Simulation and Analysis of Climate Variability on Decadal and Centennial Time Scales).

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Methane-consuming archaeobacteria in marine sediments

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Large amounts of methane are produced in marine sediments but are then consumed before contacting aerobic waters or the atmosphere¹. Although no organism that can consume methane anaerobically has ever been isolated, biogeochemical evidence indicates that the overall process involves a transfer of electrons from methane to sulphate and is probably mediated by several organisms, including a methanogen (operating in reverse) and a sulphate-reducer (using an unknown intermediate substrate)². Here we describe studies of sediments related to a decomposing methane hydrate. These provide strong evidence that methane is being consumed by archaeobacteria that are phylogenetically distinct from known methanogens. Specifically, lipid biomarkers that are commonly characteristic of archaea are so strongly depleted in carbon-13 that methane must be the carbon source, rather than the metabolic product, for the organisms that have produced them. Parallel gene surveys of small-subunit ribosomal RNA (16S rRNA) indicate the predominance of a new archaeal group which is peripherally related to the methanogenic orders Methanomicrobiales and Methanosarcinales.

We investigated sediments from a methane seep in the Eel River basin, offshore northern California (40° 47.08' N, 124° 35.30' W), an area with abundant methane hydrates at temperature–pressure conditions near their threshold of stability³. We combined a process-oriented investigation of abundances of ¹³C in individual biomarkers⁴ with culture-independent molecular phylogenetic surveys (see Methods)⁵. Hydraulic piston cores of sediments at a water depth of 521 m were retrieved by the ROV *Ventana*, operated by the Monterey Bay Aquarium Research Institute. Two seep-related samples (HPC 4, at a depth of 22 cm below the sediment surface; PC 26, 13–15 cm) and one control sample that was not near any active seeps (HPC 5, 33–36 cm) were examined. At the seep, methane bubbling into the water column is slightly enriched in ¹³C ($\delta^{13}\text{C} = -49.5\%$ relative to VPDB standard; K. Kvenvolden, unpublished data) relative to near-surface hydrates in the same region (-57.6 to -69.1% ; ref. 3). The organic matter extracted from the seep sediments contains less ¹³C than the control sample (PC 26, -33% ; HPC 5, -26.8%), presumably because products of methane-oxidizing organisms have accumulated within the microbial community.

Lipid extracts from both gas seeps contained two ether lipids that are specific to archaea and which are not present at the control site (chromatograms are shown in Fig. 1). Together with the 16:0 and 18:1 ω 9 fatty acids, both ether lipids are major components of the free-lipid fraction at gas seep PC 26, with concentrations of 0.5 and 0.35 $\mu\text{g g}^{-1}$ dry sediment, respectively (HPC 4: 0.18 and 0.04 $\mu\text{g g}^{-1}$; chromatogram not shown). Compound 1 (Fig. 1a) was identified as bis-*O*-phytanylglycerolether ('archaeol'), the ether lipid most common in archaea and especially prominent in methanogens⁶. Based on its electron-impact mass spectrum (Fig. 2), compound 2 (Fig. 1a) is a hydroxyarchaeol.

The mass spectrum of compound 2 is analogous to that of the trimethylsilyl (TMS) ether derivative of archaeol⁷. The most intense high-mass fragment ion, m/z 515, results from cleavage of the *sn*-3-phytanyl moiety. As with the TMS derivative of archaeol, a molecular ion is not present. However, the fragment at m/z 722 (displayed at m/z 723 because the precise mass is 722.697 AMU) results from the loss of TMS-OH, a process typical for TMS ethers, and substantiates the assignment of the molecular mass at 812 AMU. The hydroxy group was assigned to the *sn*-2-phytanyl chain because an identical compound was found in extracts of cultures of *Methanococcus burtonii*, a known producer of *sn*-2-hydroxyarchaeol⁸. On this basis, the structure is tentatively that shown in Fig. 2, namely 2-*O*-[3-hydroxy-3,7,11,15-tetramethyl]hexadecyl-3-*O*-[3', 7', 11', 15'-tetramethyl]hexadecyl-*sn*-glycerol (*sn*-2-hydroxyarchaeol).

Both compounds are extremely depleted in ^{13}C . The δ value for archaeol is -100‰ at both sites. Those for *sn*-2-hydroxyarchaeol are

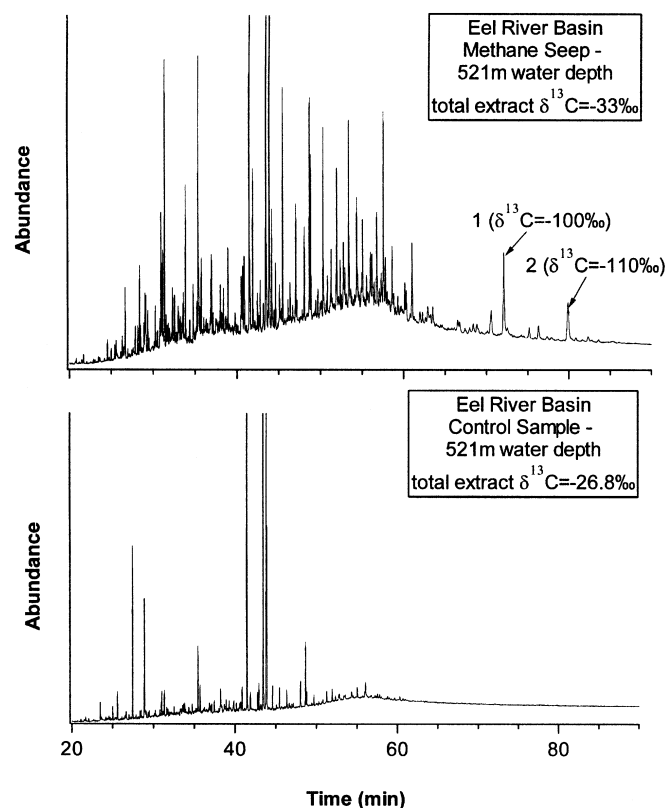


Figure 1 Reconstructed-ion-current chromatograms of trimethylsilylated total lipid extracts from **a**, a sample 13–15 cm below the sediment surface at a site of active methane seepage (PC26, Eel River Basin) and **b**, a control sample 33–36 cm below the sediment surface in the same basin but remote from any site of methane release (HPC 5). Analytical conditions for both sediment extracts were identical (similar amounts of extracted sediment, identical steps of dilution before injection into the gas chromatograph). The three large peaks which appear in both chromatograms are internal standards. The identities of peaks 1 and 2 are discussed in the text.

-110 and -105‰ at sites PC 26 and HPC 4, respectively. These isotopic compositions are not consistent with production of these lipids by methanogens that are using CO_2 as their carbon source. For CO_2 -reducing methanogens, the isotopic fractionation between CO_2 and biomass is only about half that between CO_2 and CH_4 (ref. 9), and CO_2 dissolved in porewaters in methanogenic environments is often enriched in ^{13}C (ref. 10). As a result, δ values of methanogenic biomarkers can be roughly equal to those of primary producers (for example, -30‰ ; ref. 11). The archaeal biomarkers found here, however, are depleted in ^{13}C by more than 70‰ relative to primary products. The only carbon source that is strongly enough depleted in ^{13}C is CH_4 . Therefore, the ^{13}C -depleted compounds derive from methane-consuming, rather than methane-producing, organisms. Although large, the apparent methane-lipid isotopic differences found here agree with previous observations of methanotrophic products¹¹ and with isotope fractionations associated with the growth of methanotrophic organisms in laboratory cultures¹².

Further information is provided by the results of culture-independent surveys of archaeal 16S rRNA genes in these samples. Restriction-fragment length polymorphisms (RFLP) and phylogenetic analyses of archaeal rRNA genes indicate that the diversity of archaeal types present in the seep sediments is limited. An rRNA gene library prepared from a seep sediment subscore (HPC 4, 22 cm) yielded only two major archaeal phylogenetic groups from 176 clones analysed. One of these comprises a single sequence type (Eel-BA2H11; Fig. 3). It is closely related to cultivated members of the Methanosarcinales¹³ and specifically affiliated with a group of rRNA sequences previously recovered from coastal marsh sediments¹⁴. The other rRNA sequences, accounting for 148 of the 176 archaeal clones recovered, form a cluster of unique but highly related sequences (the Eel-BA) components of group ANME-1; Fig. 3), each distinguishable by RFLP analysis and primary structure. This group, which has not been observed in other environmental surveys of archaea in either freshwater or marine sediments^{14–19}, is distinct from, but related to, the methanogenic orders Methanomicrobiales and Methanosarcinales.

The designator Eel-TA applies to components of gene libraries prepared from shallower sediments in the same core (HPC 4, 0–4 cm). Of these, 55% (79/142) of the archaeal rRNA clones are associated with the ANME-1 cluster. Most of the remaining clones are very similar to previously recovered archaeal rRNA genes from surficial coastal sediments¹⁴ and continental shelf sediments¹⁸.

Significant amounts of hydroxyarchaeols have been found before only in organisms from the order Methanosarcinales (Fig. 3). *sn*-3-Hydroxyarchaeol has been found in *Methanosaeta concilii*, whereas members of *Methanosarcina* sp. are major producers of *sn*-2-hydroxyarchaeol²⁰. Both groups produce archaeol as one of their

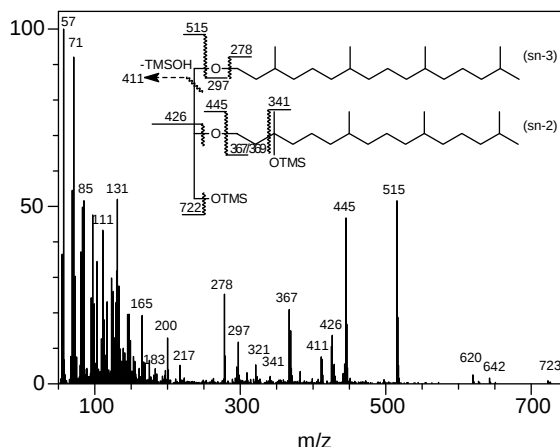
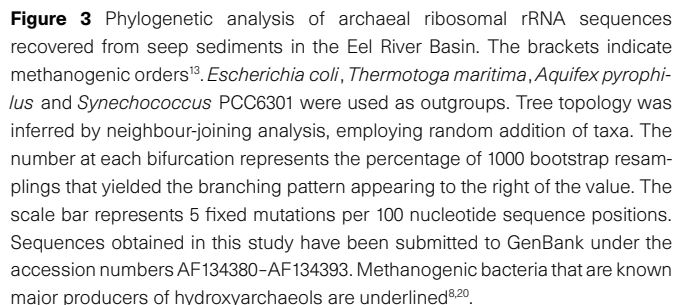


Figure 2 Electron-impact mass spectrum of peak 2, Fig. 1a. The interpretation of this spectrum, leading to the structure shown, is discussed in the text.

The complementary evidence from biomarker data and rRNA sequences indicates that a new group of archaea, phylogenetically distinct from the known orders of methanogens, is producing the observed ^{13}C -depleted lipids. There are three possible interpretations: the existence of obligately or dominantly methanotrophic archaea, the presence of methanogens functioning normally—producing methane but utilizing a carbon source that is exceptionally depleted in ^{13}C , or the presence in these sediments of methanogens that are operating in reverse.

Other ^{13}C -depleted biomarkers have been described, particularly the two irregular isoprenoids crocetane and 2,6,10,15,19-PME, which were presumed to derive from methanogens operating as methane-consumers²⁶ (V. Thiel *et al.*, and M. Elvert *et al.*, unpublished data). Biological precursors of crocetane are still unknown, but 2,6,10,15,19-PME has been found in some methanogenic bacteria²⁷. Neither compound has yet been found at the Eel River gas seeps, indicating that more than one type of microbial



assemblage involves archaea in the oxidation of methane. Future studies of environments in which methane is being oxidized anaerobically, combining biomarker techniques and 16S rRNA sequencing, are likely to reveal the identity of the crocetane producer as well as the environmental conditions that determine the growth of either one of these two (or more?) distinct assemblages.

Our results demonstrate the power of complementary information derived from molecular isotopic and phylogenetic lines of evidence. Resolution of the mechanisms by which methane is being oxidized in natural environments is very important both in terms of the global carbon budget and for reconstruction of the relationships between gas-hydrate carbon pools and concentrations of greenhouse gases in ancient atmospheres²⁸. □

Methods

Lipid analyses. We extracted free lipids using a Dionex Accelerated Solvent Extraction 200 system at 100 °C and 1000 psi with dichloromethane/methanol (99:1 v/v) as the solvent. We determined the carbon isotopic compositions of total extracts by combustion (sealed quartz tube, CuO/Ag, 850 °C, 8 h) and isotopic mass spectrometry (Micromass Optima). Individual compounds were quantified and identified using an HP 6890 gas chromatograph (GC) equipped with a J&W DB-5 (60-m length, 0.32-mm inner diameter, and 0.25-μm film thickness) capillary column and coupled to an HP 5973 mass-selective detector. Stable carbon isotopic compositions of individual compounds were determined using a Finnigan Delta Plus isotope mass spectrometer coupled to a HP 6890 GC and equipped with a column identical to that described above. Column temperatures were programmed from 40 °C (1 min isothermal) to 130 °C at a rate of 20 °C min⁻¹, and then to 320 °C (60 min isothermal) at 4 °C min⁻¹. We analysed glycerol derivatives as their trimethylsilyl ethers after reaction with BSTFA. Reported δ values are corrected for the introduction of additional carbon atoms by derivatization.

16S rRNA sequencing. We collected subcores at varying sediment depths in sterile tubes, and stored them frozen at -70 °C before nucleic acid extraction. Subsequently, thawed sediment was mixed with an equal volume of lysis buffer (10 mM Tris-HCl pH 8.3, 40 mM EDTA, 750 mM NaCl, 2% sodium dodecyl sulphate), and mechanically disrupted by bead mill homogenization with sterile 0.1 mm zirconium/silica beads. We then purified nucleic acids as previously described²⁹ by phenol/chloroform (1/1) extraction, chloroform extraction, and subsequent small-scale CsCl density gradient centrifugation. Archaeal or bacterial small-subunit rRNA genes were PCR amplified and cloned using archaeal biased (Ar 20F: TTC CGG TTG ATC CYG CCR G and AR958R: YCC GGC GTT GAM TCC AAT T) or eubacterial biased (Eub27F: AGA GTT TGA TCC TGG CTC AG and 1492R: GGT TAC CTT GTT ACG ACT T) PCR primers as described³⁰. Ribosomal RNA clones from each library (bacterial or archaeal) were initially screened by PCR amplification of rDNA plasmid inserts using M13 forward and reverse primers, and subsequent restriction endonuclease digestion using *Hae*III. We analysed restriction fragments by agarose gel electrophoresis on 2.5% NuSieve 3:1 (FMC). Ribosomal RNA clones determined to be unique by RFLP analysis were bidirectionally sequenced using infrared dye-labelled primers and a Licor automated DNA sequencer. Sequences were aligned to a database of rRNA sequences and subsequent bootstrap neighbour-joining analyses performed using Phylip, version 3.5. We calculated evolutionary distances using the Kimura 2-parameter model with a transition/transversion ratio of 2.0.

Received 19 January; accepted 11 March 1999.

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Acknowledgements. We thank C. Johnson and L. Houghton for isotopic analyses; D. Orange and K. Kvenvolden for collecting some of the sediment samples; P. D. Nichols and R. Summons for an extract of *M. burtonii*; A. Teske, J. Rullkötter, R. E. Summons, and T. Hoehler for discussions; and the officers and crew of the *Pt. Lobos*, and pilots of the ROV *Ventana*, for expert assistance. K.H. is supported by a research fellowship from the Deutsche Forschungsgemeinschaft. J.M.H., S.P.S., and laboratory expenses were supported by NASA. The Eel River expedition and E.F.D. and P.G.B. supported by the David and Lucile Packard Foundation.

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Reduced antinociception in mice lacking neuronal nicotinic receptor subunits

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Nicotine exerts antinociceptive effects by interacting with one or more of the subtypes of nicotinic acetylcholine receptors (nAChRs) that are present throughout the neuronal pathways that respond to pain^{1–5}. To identify the particular subunits involved in this process, we generated mice lacking the α4 subunit of the neuronal nAChR by homologous recombination techniques and studied these together with previously generated mutant mice

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lacking the $\beta 2$ nAChR subunit⁶. Here we show that the homozygous $\alpha 4^{-/-}$ mice no longer express high-affinity [³H]nicotine and [³H]epibatidine binding sites throughout the brain. In addition, both types of mutant mice display a reduced antinociceptive effect of nicotine on the hot-plate test and diminished sensitivity to nicotine in the tail-flick test. Patch-clamp recordings further reveal that raphe magnus and thalamic neurons no longer respond to nicotine. The $\alpha 4$ nAChR subunit, possibly associated with the $\beta 2$ nAChR subunit, is therefore crucial for nicotine-elicited antinociception.

The involvement of a particular nAChR subunit in nicotine analgesia has so far been difficult to assess. Pharmacological approaches have indicated a possible contribution of the widely expressed neuronal $\alpha 4$ subunit which co-assembles *in vivo* with the $\beta 2$ nAChR subunit (and possibly other nAChR subunits) to form high-affinity functional receptors^{5,7,8}. By gene targeting and homologous recombination, we have generated a line of mutant mice that lacks the neuronal nAChR $\alpha 4$ subunit (Fig. 1)⁶. Similar to $\beta 2^{-/-}$ mice, mice homozygous for the $\alpha 4$ mutation were born in the expected Mendelian proportion, were capable of reproduction and had no obvious physical abnormalities. In $\alpha 4^{-/-}$ mice, no detectable amounts of $\alpha 4$ messenger RNA were found throughout the brain using *in situ* hybridization (Fig. 2a)⁹. Furthermore, there was no significant difference in the expression of the mRNA of the $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 2$ and $\beta 4$ nAChR subunits or in [¹²⁵I] α -bungarotoxin binding sites (data not shown). Therefore, the disruption of the $\alpha 4$ gene did not cause compensatory changes in the amounts of other nAChR subunit mRNAs or proteins tested.

Autoradiographic ligand-binding experiments with the tritiated forms of two antinociceptive compounds, nicotine and epibatidine, showed that high-affinity binding for these compounds decreased by about 50% in heterozygous $\alpha 4^{+/-}$ mice (data not shown) and was absent in most brain regions of $\alpha 4^{-/-}$ mice. In $\alpha 4^{-/-}$ mice a reduced level of [³H]nicotine binding was detected in the interpeduncular

nucleus (IP) (Fig. 2b, c). [³H]epibatidine binding persisted at high levels in the medial habenula (MHb), superior colliculus (SC) and IP and at low levels in the substantia nigra (SN). However, the residual [³H]nicotine binding in the IP and [³H]epibatidine binding in the SN and SC of $\alpha 4^{-/-}$ mice were absent in $\beta 2^{-/-}$ mice^{6,7}, which indicates that, in these regions, subunits other than $\alpha 4$ form high-affinity nicotine- and epibatidine-binding sites *in situ*, in association with the $\beta 2$ nAChR subunit. Moreover, the pronounced reduction of [³H]nicotine and [³H]epibatidine binding in $\alpha 4^{-/-}$ and $\beta 2^{-/-}$ mice in overlapping regions indicates that these two subunits contribute to most high-affinity nicotinic-binding sites in mouse brain^{6,7,10}.

We examined several pharmacological responses of $\alpha 4^{-/-}$ -mutant mice to the nAChR agonists nicotine and epibatidine and compared these with the effect of nicotine in $\beta 2^{-/-}$ mice. Figure 3 shows the antinociceptive effects of nicotine in hot-plate and tail-flick tests, which distinguish, respectively, between a supraspinal response and a spinal reflex^{11,12}. The control latency response to painful stimuli did not differ significantly in wild-type and knockout littermates in

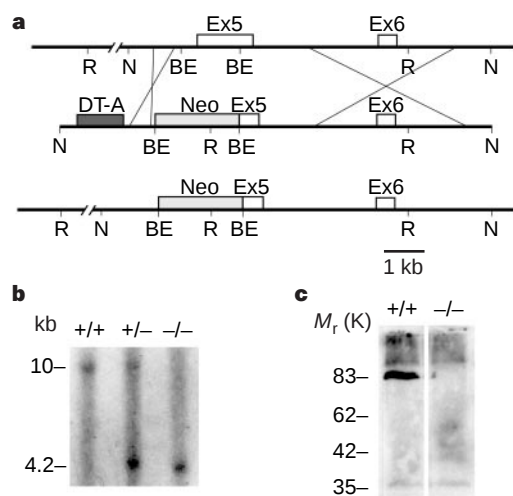


Figure 1 Targeted mutagenesis and expression analysis of the $\alpha 4$ nAChR subunit. **a**, Top, restriction map of the wild-type $\alpha 4$ -subunit gene. Ex5, and Ex6 are exon 5 and exon 6 respectively. The portion of exon 5 that was removed is indicated by the two *Bst*II restriction sites. This fragment contains three of the four putative transmembrane-spanning domains (M1, M2 and M3) and the acetylcholine binding site. Middle, targeting construct used to disrupt the endogenous $\alpha 4$ -subunit gene. Neo, the neomycin-resistance gene, replaced part of exon 5. Diphtheria toxin-A gene (DT-A)²⁶ was used to select against random integration. Bottom, structure of the recombined $\alpha 4$ gene. Restriction sites: R, *Eco*RI; N, *Nhe*I; BE, *Bst*II. **b**, Southern blot analysis of genomic DNA restricted with *Eco*RI from +/+, +/- and -/- mice. **c**, Western blot analysis of total brain extracts using a polyclonal antibody against $\alpha 4$ subunit (M.M.A.-J. *et al.*, submitted).

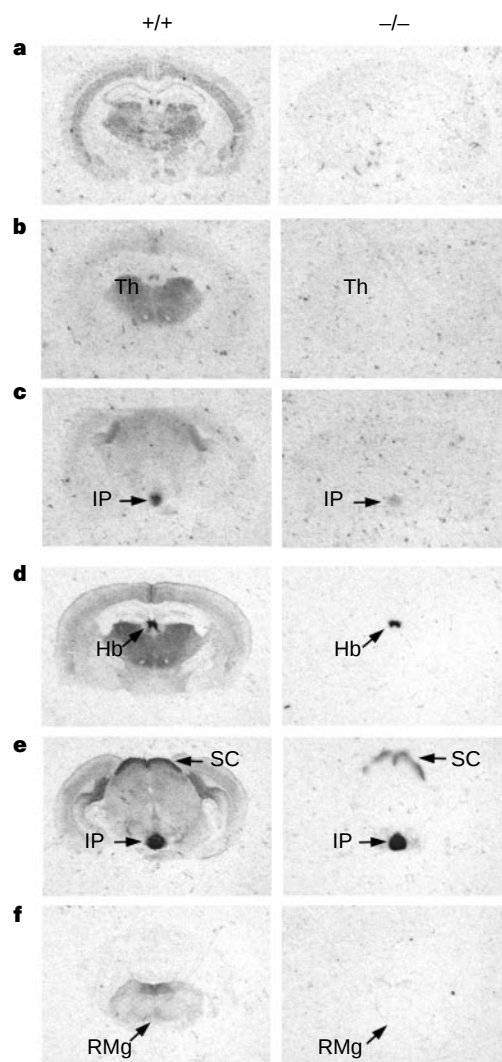


Figure 2 Expression of neuronal nAChRs in mouse brain. **a**, *In situ* hybridization using antisense oligonucleotide probes based on the $\alpha 4$ cDNA sequences. Representative mid-thalamic sections are shown. **b**, **c**, Autoradiographic mapping using [³H]nicotine to reveal the presence of high-affinity nicotine receptors in +/+ and -/- mouse brain sections at Bregma levels of 1.5 and 3.4 mm, respectively. Arrows indicate the IP and show the low levels of binding that persist in the IP in the -/- mice. **d**-**f**, Autoradiographic mapping using [³H]epibatidine in +/+ and -/- mouse brain sections at Bregma levels of 1.5, 3.4 and 5.8 mm, respectively. Note the binding that remains in the mHb, SC and IP.

either test, indicating that the endogenous activation of either the $\alpha 4$ nAChR subunit or the $\beta 2$ nAChR subunit is not essential in the perception of acute thermal nociception (Fig. 3a, d). Using the hot-plate test, $\alpha 4^{+/+}$ mice showed a dose-dependent antinociceptive response to nicotine with a median effective dose (ED_{50}) of 1.8 mg kg^{-1} (range of confidence limits $1.4\text{--}2.4 \text{ mg kg}^{-1}$), a value between the ED_{50} values for the 129 ($ED_{50} = 2.2$ ($2.0\text{--}2.5 \text{ mg kg}^{-1}$) or C57Bl/6 ($ED_{50} = 1.0$ ($0.6\text{--}1.6 \text{ mg kg}^{-1}$) strains of mice which contribute to the genetic background of the $\alpha 4^{-/-}$ mice (data not shown). For $\beta 2^{+/+}$ mice, the estimated ED_{50} value of 0.93 ($0.7\text{--}1.3 \text{ mg kg}^{-1}$) is close to that of the C57Bl/6 strain which largely contributes to the genetic background of the $\beta 2^{-/-}$ mice. Nicotine-elicited seizures occurred at 3 mg kg^{-1} nicotine in both $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice, and this dose was

therefore not tested in analgesia experiments. In contrast to wild-type littermates, $\alpha 4^{-/-}$ mice showed no antinociceptive response in the hot-plate test at all doses of nicotine tested (Fig. 3b). Their latency of response was not significantly different from control values regardless of the amount of nicotine injected. $\beta 2^{-/-}$ mice also exhibited a reduced antinociceptive response to all doses of nicotine tested (Fig. 3c). In addition, $9 \mu\text{g kg}^{-1}$ of epibatidine, a dose that showed a $91 \pm 5.8\%$ maximum possible effect (MPE) in C57Bl/6 control mice (data not shown) and $73 \pm 15\%$ MPE in $\alpha 4^{+/+}$ mice, showed no antinociceptive effect in $\alpha 4^{-/-}$ mice (Fig. 3g).

In contrast, nicotine was able to cause dose-dependent analgesia in the tail-flick test in $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice and $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice. However, in both cases the dose-response curve for the

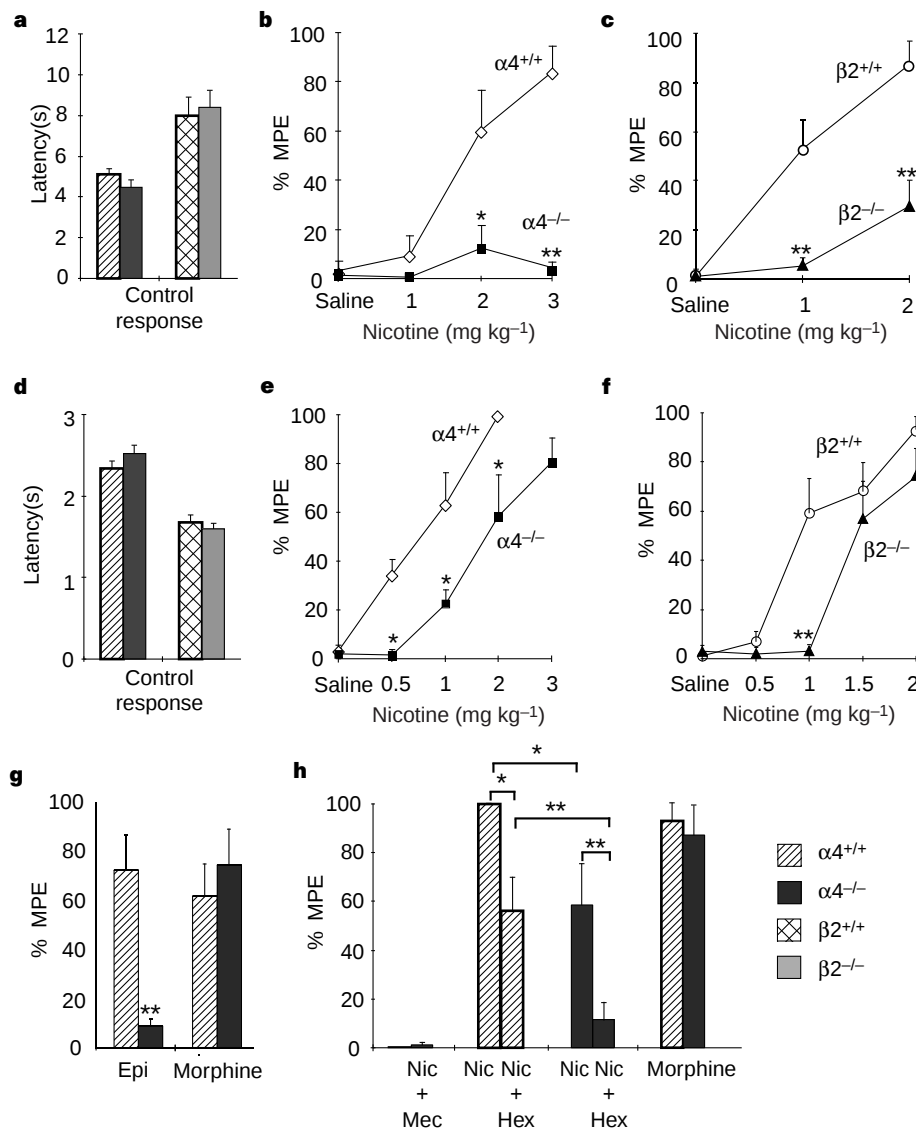


Figure 3 Antinociception of $\alpha 4$ -knockout mice in the hot-plate and tail-flick assays. **a**, Pre-test control responses of $\alpha 4^{+/+}$ ($n = 38$), $\alpha 4^{-/-}$ ($n = 40$), $\beta 2^{+/+}$ ($n = 24$) and $\beta 2^{-/-}$ ($n = 22$) mice on the hot-plate assay. **b**, Antinociception responses in the hot-plate assay to nicotine administration in $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ littermates using saline ($n = 7, 7$; $+/+$, $-/-$, respectively) and nicotine concentrations of 1 mg kg^{-1} ($n = 5, 7$), 2 mg kg^{-1} ($n = 8, 11$) and 3 mg kg^{-1} ($n = 8, 8$). **c**, Antinociception responses in the hot-plate assay to nicotine administration in $\beta 2^{+/+}$ and $\beta 2^{-/-}$ littermates using saline ($n = 5, 6$; $+/+$, $-/-$, respectively) and nicotine concentrations of 1 mg kg^{-1} ($n = 9, 7$) and 2 mg kg^{-1} ($n = 10, 9$). **d**, Pre-test control responses of $\alpha 4^{+/+}$ ($n = 40$), $\alpha 4^{-/-}$ ($n = 42$), $\beta 2^{+/+}$ ($n = 39$) and $\beta 2^{-/-}$ ($n = 36$) mice in the tail-flick assay. **e**, Antinociception responses in the tail-flick assay to nicotine administration in $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ littermates using saline ($n = 6, 5$; $+/+$, $-/-$, respectively) and nicotine concentrations

of 0.5 mg kg^{-1} ($n = 5, 5$), 1 mg kg^{-1} ($n = 11, 8$), 2 mg kg^{-1} ($n = 5, 7$) and 3 mg kg^{-1} ($n = 5$) ($\alpha 4^{-/-}$ only). **f**, Antinociception responses in the tail-flick assay to nicotine administration in $\beta 2^{+/+}$ and $\beta 2^{-/-}$ littermates using saline ($n = 6, 5$; $+/+$, $-/-$, respectively) and nicotine concentrations of 0.5 mg kg^{-1} ($n = 8, 5$), 1 mg kg^{-1} ($n = 9, 9$), 1.5 mg kg^{-1} ($n = 7, 8$) and 2 mg kg^{-1} ($n = 9, 8$). (Fischer's PLSD, $P < 0.01$). **g**, Antinociception response in the hot-plate test to $9 \mu\text{g kg}^{-1}$ epibatidine in $\alpha 4^{+/+}$ ($n = 9$) and $\alpha 4^{-/-}$ ($n = 7$) littermates and to 5 mg kg^{-1} morphine in $\alpha 4^{+/+}$ ($n = 6$) and $\alpha 4^{-/-}$ ($n = 5$). **h**, Antagonism of antinociception response in the tail-flick assay to 2 mg kg^{-1} nicotine in $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ littermates using 5 mg kg^{-1} mecamylamine ($n = 5, 5$) and 5 mg kg^{-1} hexamethonium ($n = 8, 7$). Antinociception response in the tail-flick assay in $\alpha 4^{+/+}$ ($n = 6$) and $\alpha 4^{-/-}$ ($n = 5$) to 5 mg kg^{-1} morphine. Values on the y-axis expressed as mean $\pm$ s.e.m. *, $P < 0.05$; **, $P < 0.01$. Mec, Mecamylamine; Hex, Hexamethonium.

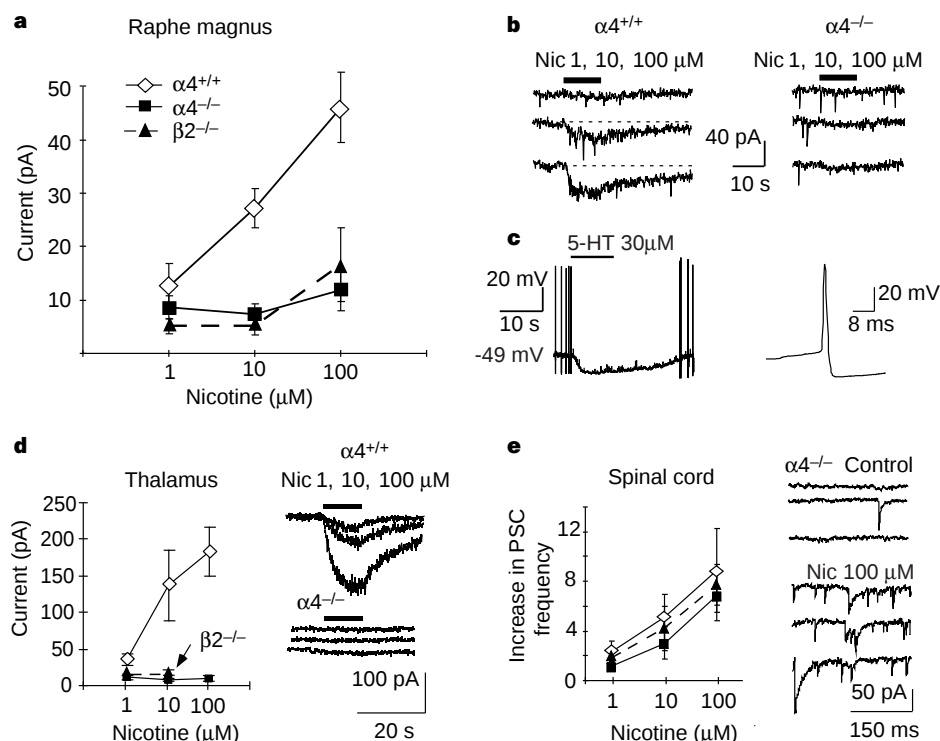


Figure 4 Patch-clamp recordings of nicotine-evoked currents in raphe magnus, thalamus and dorsal horn of the spinal cord of $\alpha 4^{+/+}$, $\alpha 4^{-/-}$ and $\beta 2^{-/-}$ mice. **a**, Dose-response curve for nicotine in $\alpha 4^{+/+}$, $\alpha 4^{-/-}$ and $\beta 2^{-/-}$ raphe magnus serotonergic neurons. In $\alpha 4^{+/+}$ mice, nicotine concentrations of 10 and 100 μM elicited a significant current in nine out of ten neurons. The non-responding neuron in $\alpha 4^{+/+}$ was not included in the dose-response curve. **b**, Nicotine (1–100 μM) elicits an inward current in serotonergic raphe magnus neurons recorded in whole-cell configuration (voltage clamp) in $\alpha 4^{+/+}$ but not in $\alpha 4^{-/-}$ or $\beta 2^{-/-}$ mice. **c**, Serotonin elicits the typical hyperpolarization in serotonergic neurons in the raphe magnus¹⁷ in $\alpha 4^{+/+}$ ($n = 10$), $\alpha 4^{-/-}$ ($n = 9$) and $\beta 2^{-/-}$ ($n = 7$) mice. A representative trace of the response to serotonin (left) and a representative action potential of these neurons (right) are shown. **d**, Dose-response curve for nicotine in the $\alpha 4^{+/+}$

($n = 15$ neurons), $\alpha 4^{-/-}$ ($n = 11$ neurons) and $\beta 2^{-/-}$ ($n = 10$ neurons)⁶ antero-ventral thalamus (left). Currents elicited by nicotine in $\alpha 4^{+/+}$ thalamic neurons in whole-cell configuration (top, right). No nicotine-elicited current is observed in $\alpha 4^{-/-}$ thalamic neurons for 1, 10 or 100 μM nicotine (bottom, right). **e**, Dose-response curve for nicotine-induced increase of PSC frequency in the $\alpha 4^{+/+}$ ($n = 8$), $\alpha 4^{-/-}$ ($n = 9$) and $\beta 2^{-/-}$ ($n = 12$) spinal dorsal horn neurons (layers I–III) (left). Representative traces from an $\alpha 4^{-/-}$ neuron before (top, right) and during (bottom, right) application of 100 μM nicotine are shown. Values for all dose-response curves correspond to mean $\pm$ s.e.m. of currents recorded at -60 mV. Each point corresponds to the average of four to nine measures. Electrode resistance was 1–2 M Ω for experiments in the raphe magnus and thalamus and 3–5 M Ω in the spinal cord.

mutant mice was shifted to the right ($\alpha 4^{+/+}$ $\text{ED}_{50} = 0.7$ (0.5–1.0) mg kg^{-1} compared with $\alpha 4^{-/-}$ $\text{ED}_{50} = 1.6$ (1.2–2.1) mg kg^{-1} ; $\beta 2^{+/+}$ $\text{ED}_{50} = 1.0$ (0.8–1.1) mg kg^{-1} compared with $\beta 2^{-/-}$ $\text{ED}_{50} = 1.5$ (1.3–1.7) mg kg^{-1}) (Fig. 3c, f). Thus, both the $\alpha 4$ and the $\beta 2$ nAChR subunit contribute to the antinociceptive effects of nicotine, although to a larger extent in the hot-plate test, which primarily reflects activation of supraspinal regions.

There were no significant differences between $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice after morphine administration in both the hot-plate and tail-flick tests (Fig. 3g, h), indicating that opiate analgesia does not depend on activation of $\alpha 4$ -containing nAChRs^{13,14}. In addition, there was no significant difference in non-habituated locomotor activity after injection of 1 or 2 mg kg^{-1} nicotine (data not shown), indicating that the response to nicotine of $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice and $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice in tests for analgesia does not depend only on differences in nicotine-elicited effects on the locomotor system.

The thalamus, raphe magnus, pedunculopontine tegmental nuclei and the dorsal horn of the spinal cord are important in the nicotinic antinociceptive pathways^{15,16}. Equilibrium-binding experiments showed the loss of high-affinity nicotine- and epibatidine-binding sites in these areas in $\alpha 4^{-/-}$ and $\beta 2^{-/-}$ mice (Fig. 2b–f)^{6,7}. Consistent with this finding, patch-clamp recordings from serotonergic neurons in the raphe magnus (identified by their response to serotonin)¹⁷ and neurons in the thalamus showed a loss of nicotine-elicited currents in $\alpha 4^{-/-}$ and $\beta 2^{-/-}$ mice (Fig. 4a–d)⁶, indicating a contribution of the $\alpha 4$ and $\beta 2$ nAChR subunits to

functional receptors in areas implicated in supraspinal nicotine-elicited antinociception^{13,16}. In contrast, neurons in the superficial layers (I–III) of the dorsal horn of the spinal cord of $\alpha 4^{-/-}$ and $\beta 2^{-/-}$ mice continued to display a nicotine-elicited, dose-dependent augmentation of the frequency of postsynaptic currents (PSCs) (Fig. 4e).

We then investigated the reasons why the $\alpha 4^{-/-}$ mutation only partially reduced the antinociceptive response to nicotinic agonists in the tail-flick test. First, the administration of mecamylamine, a nicotinic antagonist that acts both centrally and peripherally, completely blocked nicotine-elicited antinociception in the tail-flick test (Fig. 3f). Furthermore, previous studies have shown that 5 mg kg^{-1} hexamethonium, a peripheral antagonist that penetrates the blood–brain barrier quite poorly¹⁸, partially blocks the tail-flick response in rats and mice^{13,19}. Indeed, in wild-type mice $\sim 50\%$ of the tail-flick response to 2 mg kg^{-1} nicotine was blocked by hexamethonium, in contrast to 80% in $\alpha 4^{-/-}$ mice (Fig. 4f). The almost complete block by hexamethonium of the residual antinociception in the knockout mice indicates a contribution of peripheral, non- $\alpha 4$ receptors. To identify these peripheral receptors, we performed polymerase chain reaction with reverse transcription (RT-PCR) on complementary DNA from the dorsal root ganglion using degenerative oligonucleotide primers. We observed the expression of $\alpha 3$, $\alpha 6$, $\alpha 7$, $\beta 2$ and $\beta 4$ subunits in both $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice, the $\alpha 4$ subunit being detected only in the $\alpha 4^{+/+}$ mice (data not shown). Immunoprecipitation has shown²⁰ that most of the nicotinic

receptors in sensory ganglion are composed of $\alpha 3$ and $\beta 4$ subunits, whereas a minority contains $\alpha 4$ and $\beta 2$ subunits. Thus, in the $\alpha 4^{-/-}$ mice $\alpha 3$ - and $\beta 4$ -subunit-containing nAChRs are likely candidates to mediate the peripheral effects of nicotine in the tail-flick test.

In conclusion, disruption of the $\alpha 4$ and the $\beta 2$ nAChR gene demonstrates that these receptor subunits are an important, although not exclusive, component of the nicotinic pain pathways and most likely cooperate to mediate the antinociceptive effect of nicotine. Specific pharmacological compounds that target these receptors may prove to be therapeutically useful for analgesia. □

Methods

Generation of mutant mice. Mouse genomic DNA encoding exon 5 and 6 of the $\alpha 4$ subunit was isolated from a λ DASH II male 129 mouse strain genomic library. A DNA construct for homologous recombination was produced by replacing a 1.3 base pair (bp) fragment consisting of a portion of exon 5 and about 400 bp of the upstream intron sequence with a cassette encoding for the neomycin-resistance gene. Transfected embryonic stem-cell colonies that survived after selection with neomycin were subcloned and PCR was used to identify homologously recombined versus randomly integrated DNA. Four positive colonies were isolated and injected into mouse blastocysts. Chimaeric offspring were mated to non-agouti, C57Black/6 mice. Transmission of the mutant $\alpha 4$ gene was indicated by the colour coat marker in approximately 1 out of every 100 of the progeny followed by PCR analysis of DNA isolated from the tail. The positive F1 progeny were crossed with C57Bl/6 mice and their F2 heterozygous offspring were interbred to generate homozygote $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ littermates. For the generation of $\beta 2^{+/+}$ and $\beta 2^{-/-}$ littermates, F7 (backcrossed with C57Bl/6 for seven generations) heterozygote breeders were used.

Mapping of the neuronal nAChR mRNA in mouse brain. *In situ* hybridization was performed as described⁵. Oligonucleotides used differing from those previously described are as follows: $\alpha 4$ (mouse) 5'-CTGGGCA-CAGCATTCTACTTCTGGTGTGTAGGGTCCACGGC-3', 5'-GAAGTC-CAGTTGGTCCACACGGCTGTGCATGCTCACCAA-3'.

Western blot analysis. Whole brain extracts from $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice were homogenized in five volumes of boiling lysis buffer (1% SDS, 10 mM Tris-Cl, pH 7.4) and centrifuged at 550g for 10 min. The supernatant was collected, divided into aliquots and frozen at -80°C until use. Samples were run on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The $\alpha 4$ -subunit antibody was from Santa Cruz Biotechnology and purified as described (M.M.A.-J. *et al.*, submitted).

Equilibrium binding. Receptor autoradiography was performed as described^{7,10}. [³H]nicotine and [³H]epibatidine were obtained from Amersham.

Behavioural analysis. Antinociception was assessed by the tail-flick method as described²¹. A control response (2–6 s) was determined for each animal before treatment and a test latency was determined after drug administration. To minimize tissue damage, a maximum latency of 10 s was imposed. Antagonism studies were done by pretreating the mice subcutaneously with the nicotinic antagonists, mecamylamine or hexamethonium, 10 min before administration of nicotine. The animals were tested 5 min after subcutaneous administration of agonist or 15 min after administration of 5 mg kg⁻¹ morphine. Antagonists were administered subcutaneously 15 min before nicotine administration.

Mice were also examined using the hot-plate method (at a temperature of 55°C). A control response was determined for each animal before treatment. A cut-off time was imposed of 20 s for the $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice and 60 s for $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice. The nociceptive endpoints in the hot-plate test were hind paw-licking, jumping or rapid thumping of the hind paw. Mice were tested 5 min after subcutaneous injections of nicotine or epibatidine or 20 min after 5 mg kg⁻¹ morphine injection for the dose-response evaluation. Some mice were used for both the hot-plate and the tail-flick tests and some $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice injected with saline or low doses of nicotine were used twice on the tail-flick test. Antinociceptive response was calculated as %MPE, where %MPE = [(test – control)/(cut-off – control)] × 100. Experimenters were blind as to the genotype of the mice. After the tail-flick or hot-plate assay, some mice were tested for locomotor activity as described²².

Data were analysed by an analysis of variance followed by Fisher's post-hoc least significant difference (PLSD) multiple comparison test. The null

hypothesis was rejected at the 0.05 significance level. ED₅₀ values with 95% confidence limits for antinociception data were calculated by unweighted least-squares linear regression, as described²³. Potency ratios were used to compare ED₅₀ curves as described²³. A Student's *t*-test was used for comparison between individual groups. The ethical guidelines of the International Association for Study of Pain were followed²⁴.

Patch-clamp recording. Electrophysiological experiments were done as previously described²². Coronal slices were taken from animals at postnatal day 7–11 (P7–11) for the raphe magnus, P7–14 for the thalamus, and P6–8 for the spinal cord. For the raphe magnus, the intracellular recording solution contained 130 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, 10 mM EGTA, 4 mM ATP-Na₂, 0.4 mM GTP-Na, 10 mM HEPES, pH 7.2. For the spinal cord and thalamus recordings, the intracellular solution contained 140 mM CsCl, 2 mM MgCl, 10 mM BAPTA, 4 mM ATP-Na₂, 10 mM HEPES, pH 7.2. Presynaptic responses were analysed as in ref. 25.

RT-PCR. Extraction of total RNA from freshly frozen dorsal root ganglion and spinal cord as a positive control for subunits not expressed in the dorsal root ganglion from $\alpha 4^{+/+}$ and $\alpha 4^{-/-}$ mice was performed using the RNeasy kit (Qiagen, Germany). First-strand cDNA was synthesized with SuperscriptII (Gibco BRL) with random hexamer primers. PCR amplification of nAChRs was done using the following forward oligonucleotide primers: $\alpha 2$ 5'-CTTCTTCACGGGCACTGTGCACTGGGTG-3', $\alpha 4$ 5'-GTTCTATGACGGAA-GGGTGCAGTGGACA-3', $\alpha 3$ 5'-ACTCAAGTACACAGGAGAAGTGACT-3', $\alpha 6$ 5'-TCTTAAGTACGATGGGTGATAACC-3', $\alpha 5$ 5'-GATAAAGATTATACGT-GTTCCTTCG-3', $\beta 3$ 5'-AATTAATTCGATAAAGGTTCATCA-3', $\beta 2$ 5'-CTTCTATTCCAATGCTGTGGTCTCTATG-3', $\beta 4$ 5'-TGTCTACACCAAC-GTGATTGTGCGTTCCA-3', $\alpha 7$ 5'-GTGGAACATGTCTGAGTACCCCG-GAGTGAA-3'; and the following reverse oligonucleotide primers: $\alpha 2/4$ 5'-GGGATGACCAGCGAGGTGGACGGGATGAT-3', $\alpha 3/6$ 5'-TGGTCTCKGT-AATCACCAGSAGAAAGACAGT-3', $\alpha 5$ 5'-GGCAAAGACAGTCACCATAAT-GGATAGGG-3', $\alpha 7$ 5'-GAGTCTGCAGGCAGCAAGAATACCAGCA-3', $\beta 2/\beta 4$ 5'-AGCGGTACGTCGAGGGAGGTGGGAGG-3', $\beta 3$ 5'-GACCGTGAGAAA-AGACAACCCAGG-3'. cDNA was amplified for 35 cycles of one minute at each of 96°C, 60°C and 72°C.

Received 22 December 1998; accepted 11 March 1999.

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Acknowledgements. The authors thank S. Brown and M. Picciotto for their contribution to this project; M. Zoli for scientific discussions; N. Bordes for help with experiments; and P. Parra, S. Edelstein and R. Klink for critical reading of the manuscript. This research was supported by grants from the Collège de France, the Association Française contre les Myopathies, Reynolds Pharm., EEC Biotech and Biomed Programs, the Council for Tobacco Research, the National Alliance for Research on Schizophrenia and Depression, and NIH (for M.I.D.).

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***Tbx5* and *Tbx4* genes determine the wing/leg identity of limb buds**

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Much progress has been made in understanding limb development^{1–3}. Most genes are expressed equally and in the same pattern in the fore- and hindlimbs, which nevertheless develop into distinct structures^{3–8}. The T-box genes *Tbx5* and *Tbx4*, on the other hand, are expressed differently in chick wing (*Tbx5*) and leg (*Tbx4*) buds^{9–12}. Molecular analysis of the *optomotor blind* gene¹³, which belongs to the same family of transcription factors, has revealed that this gene is involved in the transdetermination of *Drosophila* wing and leg imaginal discs¹⁴. In addition, expression of *Tbx5* and *Tbx4* correlates well with the identity of ectopic limb buds induced by fibroblast growth factor^{4,5,15–19}. Thus, it is thought that *Tbx5* and *Tbx4* might be involved in determining limb identity. Another candidate is the *Pitx1* gene, which encodes a bicoid-type homeodomain transcription factor that is expressed in leg buds^{20,21}. Here we determine the importance of these factors in establishing limb identity.

To introduce foreign complementary DNA efficiently into chick embryonic cells, we adopted a novel *in ovo* electroporation system²². In a conventional replication-competent avian sarcoma (RCAS) retrovirus system, there is a time lag (of about 16 h) between injection of the virus and full gene expression²³. This could prevent the conversion of wing/leg identity, because limb identity seems to be determined and fixed at early stages (stages 10 to 12)^{24,25}. To overcome this problem, we electroporated RCAS retrovirus plasmids directly into the prospective limb-bud fields. In this system, gene expression starts immediately after electroporation (within 2 h²²). In addition, subsequent production and infection of the recombinant retrovirus fix, expand and maintain the expression in limb buds. We used this system to express *Tbx5*/*Tbx4* ectopically in limb fields at stages 7 to 9.

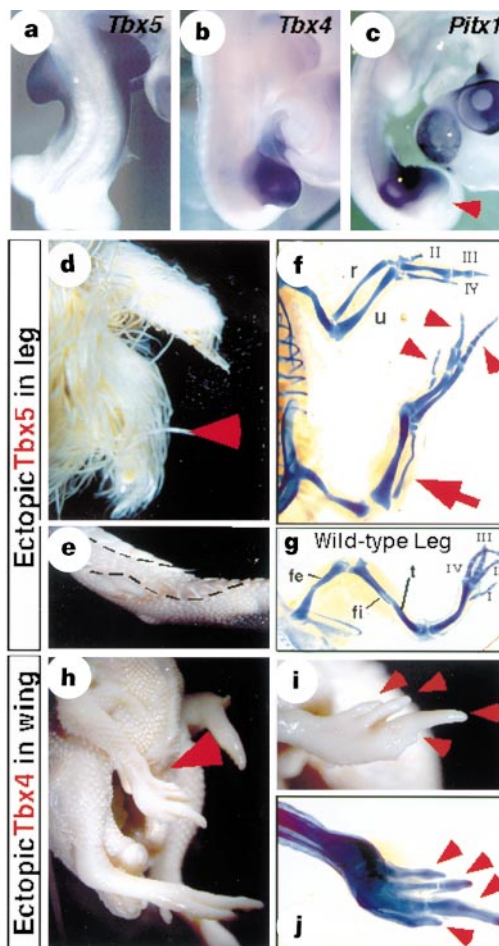


Figure 1 Misexpression of *Tbx5* and *Tbx4* converts the wing/leg identity of limb buds. **a–c**, Normal expression patterns of *Tbx5* (**a**), *Tbx4* (**b**) and *Pitx1* (**c**) genes at stage 23. **d–f**, *Tbx5* misexpressed leg. Leg ectoderm is covered by feathers (**d**, red arrowhead). Fibula is elongated and makes a joint at its distal end like a normal ulna (**f**). **e**, Partial scale-to-feather conversion in the area indicated by broken lines. **g**, Skeletal pattern of the normal leg. **h–j**, *Tbx4* misexpressed wings. The wing was elongated and four separated digits were formed with claws (red arrowheads, **i**). Four digits and a thin ulna are observed (red arrowheads, **j**). r, radius; u, ulna; fe, femur; fi, fibula; t, tibia; I–IV, digits.

When a fusion construct containing *Tbx5* and the enhanced green fluorescent protein gene (RCASBP–*Tbx5*–EGFP) was electroporated into the leg fields of chick embryos, several mesodermal and ectodermal phenotypes were observed in 15% ($n = 519$) of electroporated legs (Fig. 1d–f). The fibula, which is normally short (Fig. 1g) and does not articulate with the metatarsal bones, was elongated, and clearly articulated with metatarsal bones (red arrow in Fig. 1f), although it did not articulate correctly with the femur and was thinner than a normal ulna. The number of phalanges was different from in a normal wing, but there were only three digits, as in normal wings (arrowheads in Fig. 1f). Thus, misexpression of *Tbx5* in the leg can induce wing-like skeletal patterns, albeit partially. Ectodermal phenotypes were also converted. Figure 1d shows an electroporated leg which is completely covered in feathers (red arrowhead), without any scales. Partial scale-to-feather conversion is shown in Fig. 1e: in this case, scales were completely replaced with feathers in the leg area (dashed lines). These results indicate that misexpression of the *Tbx5* gene in the embryonic chick leg induces a partial wing-like morphology, with three digits, elongation of the fibula and scale-to-feather conversion.

We also induced misexpression of *Tbx4* in the wing fields. When RCASBP–*Tbx4*–EGFP was electroporated into the prospective

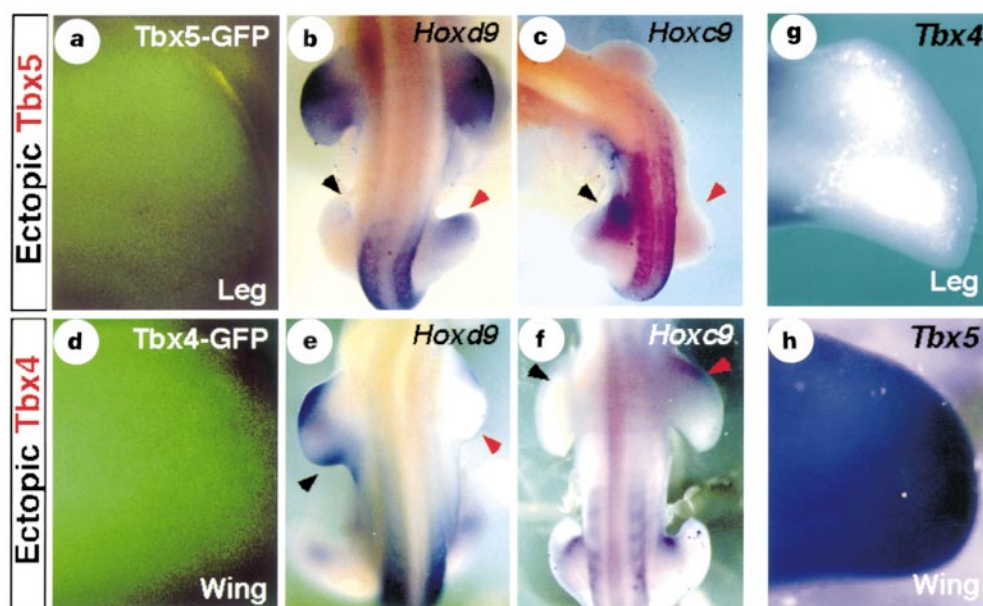


Figure 2 *Tbx5* and *Tbx4* regulate *Hoxc9/d9* expression in the developing limb buds. **a**, GFP fluorescence signal derived from introduced *Tbx5*-GFP fusion proteins in leg bud. In such leg buds, wing-specific *Hoxd9* was induced (**b**), and leg-specific *Hoxc9* was suppressed (**c**) (red arrowheads). Note the normal expression patterns of *Hoxd9* and *Hoxc9* in the wing buds and the left leg buds

(black arrowheads). **d**, GFP fluorescence derived from *Tbx4*-GFP fusion proteins in wing bud. In such wing buds, wing-specific *Hoxd9* was suppressed (**e**) and leg-specific *Hoxc9* was induced (**f**) (red arrowheads). **g**, **h**, Misexpression of *Tbx5* suppresses the endogenous *Tbx4* (**g**), whereas misexpression of *Tbx4* does not affect *Tbx5* expression (**h**).

wing region, unique morphological alterations were observed (5%; $n = 350$). As shown in Fig. 1h–j, four distinct digits were formed, although a thin ulna articulates at its distal end (Fig. 1j). Claws were formed on these digits, as on normal legs (Fig. 1i). Feather-to-scale conversion was also seen on the distal limb surface, although the proximal limb surface was covered by feather buds (Fig. 1h, i). These results indicate that misexpression of the *Tbx4* gene in the wing can induce a leg-like appearance with four distinct digits, claw formation, feather-to-scale conversion and thin ulna formation.

In these misexpression studies, we obtained broad expression of the electroporated plasmids in both wing and leg; the introduced *Tbx5*/*Tbx4* genes were misexpressed in both limb buds, because the electrodes we used in these studies span both the prospective wing and leg fields of stage 7–9 embryos. Furthermore, misexpression of irrelevant genes (alkaline phosphatase and green fluorescent protein (GFP)) did not induce consistent morphological changes in limb buds. These observations indicate that the phenotypic alterations induced by electroporation of the *Tbx5* and *Tbx4* genes are specific changes and that the nonspecific effects of our electroporation are minimal.

To analyse further the mechanism of phenotypic conversion of limb-bud identity, we examined the expression of several *Hox* genes which are expressed in a leg- (*Hoxc9*) or wing- (*Hoxd9*) specific manner^{6–8}. We monitored the expression of *Tbx* genes by observing GFP signals derived from fusion products of introduced *Tbx5*- and *Tbx4*-EGFP genes. As shown in Fig. 2a and d, *Tbx5* and *Tbx4* were misexpressed in the leg and wing buds, respectively. In such legs, wing-specific *Hoxd9* was clearly induced (53%, $n = 31$; Fig. 2b, red arrowhead) and leg-specific *Hoxc9* was repressed (26%; $n = 23$; Fig. 2c, red arrowhead) by misexpression of *Tbx5*. In contrast, leg-specific *Hoxc9* was induced, albeit weakly, in wings where introduced *Tbx4* was expressed (16%, $n = 26$; Fig. 2f, red arrowhead) and wing-specific *Hoxd9* was suppressed (8%, $n = 25$; Fig. 2e, red arrowhead). These data indicate that expression of the *Hoxc9* and *Hoxd9* genes correlates well with the morphological changes seen in the limb buds, and that some *Hox* genes are the downstream targets of *Tbx5*/*Tbx4* in chick limb buds.

To explore the regulatory hierarchy between *Tbx4* and *Tbx5*, we

examined the expression of endogenous *Tbx4* in legs where *Tbx5* was misexpressed, and vice versa. When *Tbx5* was misexpressed in leg buds, expression of endogenous *Tbx4* was suppressed (Fig. 2g). In a distal leg region, endogenous *Tbx4* transcripts were no longer detected by whole-mount *in situ* hybridization, indicating that misexpression of *Tbx5* in the leg can completely convert the expression patterns of *Tbx5* and *Tbx4* by suppressing endogenous *Tbx4* expression. In contrast, misexpression of *Tbx4* in wing did not affect endogenous *Tbx5* expression (Fig. 2h), demonstrating the coexistence of both *Tbx5* and *Tbx4* in the same limb.

We also examined the endogenous expression of *Pitx1* in these limb buds. Expression of *Pitx1* was not affected by the forced expression of *Tbx5* or *Tbx4*; *Pitx1* was expressed normally in the *Tbx5*-expressing leg and was not induced in the *Tbx4*-expressing wing, regardless of morphological changes (data not shown).

Because *Tbx4* cannot suppress endogenous *Tbx5* expression, both *Tbx5* and *Tbx4* are expressed simultaneously in the wing. To assess the effect of *Tbx4* misexpression, we exploited the modified fibroblast growth factor-8 (FGF8)-induced extra-limb-bud system. Extra limbs show wing-like appearance when induced at the level of somite 21, whereas leg-like structures arise when induced at the level of somite 25 (refs 4, 5, 16–19). In addition, classical transplantation experiments demonstrated that leg-like structures were formed when leg mesenchyme cells, which express *Tbx4*, were implanted into wing buds²⁶. We implanted, at the level of somite 21, mixed aggregates of FGF8-expressing chicken embryonic fibroblast (CEF) cells and wing mesenchyme cells in which *Tbx4* was misexpressed. These wing mesenchyme cells were prepared from stage-20 embryos, then transfected with the RCASBP-*Tbx4*-EGFP plasmid, and passaged to allow propagation of the recombinant virus. Endogenous *Tbx5* expression was decreased or lost *in vitro* (data not shown). This method allowed us to reconstitute extra limb buds that express *Tbx4*. Although *Tbx5* can be induced in such limb buds when they are formed near the wing, *Tbx4* expression should predominate in the initial phases. Furthermore, the implanted cells produce the replication-competent *Tbx4* virus which infects the surrounding host cells.

As reported, implantation of FGF8-producing CEF cells alone at

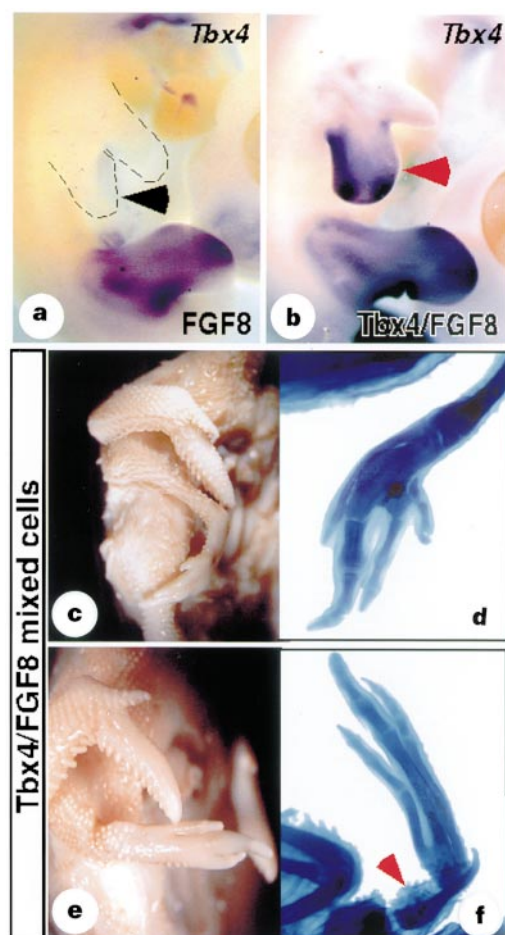


Figure 3 Limb identities of the extra limbs induced in the flank by the mixed-cell aggregates of *Tbx4*/FGF8. Mixed-cell aggregates of chicken embryonic fibroblast cells producing FGF8 and wing mesenchyme cells which express *Tbx4* were implanted in the flank to reconstitute extra limb buds. In such extra limb buds, robust expression of *Tbx4* was observed (red arrowhead, **b**) whereas *Tbx4* was not expressed in extra limb buds when FGF8 cells alone were implanted (black arrowhead, **a**). 10 days after implantation, leg-like extra limbs were formed (**c**, **e**). These extra limbs show leg-like skeletal patterns (**d**, **f**). In the zeugopod, only tibia-like bone was observed (red arrowhead, **f**).

the level of somite 21 resulted in the induction of wing-like extra limbs^{15–19}. However, when mixed aggregates of the FGF8 cells and *Tbx4*-positive wing cells were implanted at the same somite level, leg-like limbs were formed; although these limbs are bent and short (Fig. 3c and e; 45%, $n = 20$), they have three or four separated digits with scales and claws. Their leg identity was also confirmed by the skeletal patterns, showing leg-like phalange formation, elongated tibia-like bone and suppression of ulna-like bone formation (Fig. 3d and f). As expected, *Tbx4* was expressed in such limb buds (Fig. 3b), whereas FGF8 cells alone did not induce *Tbx4* (Fig. 3a). When mixed aggregates of FGF8 cells and normal wing cells (*Tbx4*-negative) were implanted, only wing-like extra limbs developed (data not shown).

We found that both host and donor mesenchyme cells contributed to the formation of extra limbs. However, at the initial stages, implanted mesenchyme cells primarily contributed to the extra limb buds, so expression of *Tbx4* predominated. We speculate that the initial expression pattern of *Tbx* genes is critical for the determination of limb identity, because electroporation of *Tbx* genes in late stages does not convert limb identity well, whereas early electroporation (stage 7 to 9) induces clear morphological changes. Hence, initial *Tbx4* expression induces leg-like morphology even though

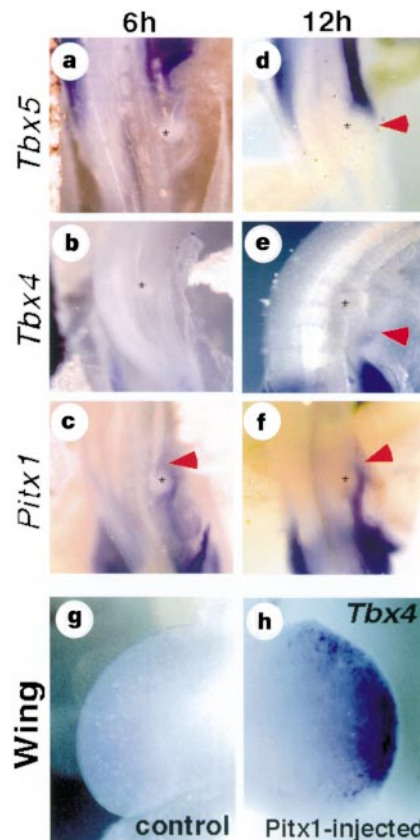


Figure 4 The regulatory network of the *Tbx5*, *Tbx4* and *Pitx1* genes. **a–f**, Aggregates of FGF8 cells (indicated by asterisks) were implanted in the flank of stage 13–15 embryos at the level of somite 23. 6 h after implantation, *Pitx1* was induced in the tissue surrounding implanted cells (**c**) while *Tbx5* and *Tbx4* were expressed only after 12 h (**a** and **d**, **b** and **e**, respectively). Note the gap between the expression domains of *Tbx4* and *Pitx1* (**e**, **f**). When *Pitx1* was misexpressed in the right wing bud, *Tbx4* was induced (**h**) whereas the normal left wing bud does not express *Tbx4* (**g**).

Tbx5 is induced in such extra limb buds in later stages. We conclude that *Tbx4* could act as a determinant of leg identity and that *Tbx4* induces more drastic conversion when its expression predominates at the initial stages of limb development.

The *Pitx1* gene is first seen by stage 11 in the posterior lateral plate mesoderm; thus, its expression precedes that of *Tbx4*. The domain of *Pitx1* expression is broader than that of *Tbx4*, which is confined to the prospective leg-bud field^{9–12,20–21}. Thus, *Pitx1* might induce *Tbx4* at early stages. To test this possibility, we induced extra limb buds by implanting FGF8-producing cells, and analysed the time course of the induction of *Tbx5*/*Tbx4* and *Pitx1*. We implanted the FGF8 cells alone in the lateral plate mesoderm at the level of somite 23 (refs 16–19). As shown in Fig. 4c, FGF8 cells induce *Pitx1* expression within 6 h of implantation, and *Tbx5*/*Tbx4* expression is induced 12 h later (Fig. 4d, e). This serial expression of genes seems to agree with the time course of the endogenous expression of *Tbx5*/*Tbx4* and *Pitx1*; thus, the expression of *Pitx1* precedes that of *Tbx4* even in FGF8-induced extra-limb buds. However, *Tbx4* was induced in a region which does not directly contact FGF8 cells (Fig. 4e), whereas *Pitx1* was expressed in tissues surrounding the implanted FGF8 cells (Fig. 4c, f). This gap between expression domains suggests that *Tbx4* might not be a direct target of *Pitx1*. We then misexpressed *Pitx1* in

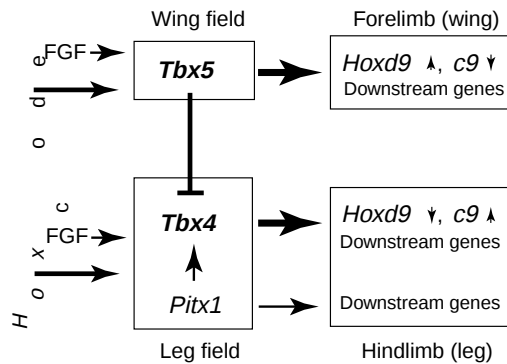


Figure 5 Interactions regulating the wing/leg identity of limb buds. Hox code and FGF expression in the lateral plate mesoderm specify and select the expression of *Tbx5* and *Tbx4/Pitx1* in the prospective wing and leg buds, respectively. *Pitx1* acts as a *Tbx4* inducer, and *Tbx5* represses *Tbx4*. Hence, mutually exclusive expression patterns of *Tbx5* and *Tbx4* are established. Then *Tbx5* induces *Hoxd9* and represses *Hoxc9* in the wing bud. Conversely, *Tbx4* represses *Hoxd9* and induces *Hoxc9* in the leg bud. *Tbx5*, *Tbx4* and *Pitx1* also induce their own sets of downstream target genes.

the wing buds. As shown in Fig. 4h, *Tbx4* is induced in the distal portion of wing buds, whereas the normal wing bud does not express *Tbx4* (Fig. 4g). Thus we conclude that *Pitx1* acts indirectly as a *Tbx4* inducer.

Limb identity is determined at around stage 10–12 (refs. 24, 25), but expression of *Tbx5/Tbx4* is first seen at stage 12 (refs 9–12). This time lag suggests the involvement of additional signals that mediate *Tbx5/Tbx4* expression. We have found that *Pitx1* induces *Tbx4*. In addition, *Hox9* genes are expressed in the lateral plate mesoderm⁸. It is possible that the *Hox* code in the lateral plate mesoderm could select the *Pitx1* and *Tbx* genes, as the expression of some *Hox* genes precedes that of *Pitx1* and *Tbx5/Tbx4*. As *Tbx5* and *Tbx4* can induce or suppress *Hox9* genes, the tight interplay among *Pitx1*, *Tbx* and *Hox* genes in both the lateral plate mesoderm and limb buds is important for the correct specification of limb identity. However, the interplay between the *Tbx* and *Hox* genes does not necessarily imply a feedback loop, because *Hox* genes play distinct roles in different phases during limb development.

As shown in Fig. 1c, *Pitx1* expression starts to fade out in the distal and posterior portion of leg buds from stage 23. In later stages, this gene is expressed in relatively restricted areas, whereas expression of *Tbx4* is maintained in the entire leg mesenchyme cells. These observations indicate that *Pitx1* might not be involved in the maintenance of *Tbx4* expression. After being triggered by retinoic acid, expression of *Hoxb1* is maintained through the autoregulatory element in its own promoter^{27,28}. Therefore it is possible that a similar auto-regulatory mechanism might operate to maintain and fix *Tbx5/Tbx4* expression in limb buds. If so, the ability of *Tbx5* to suppress *Tbx4* could be explained by the crosstalk between the *Tbx5* and *Tbx4* proteins on the autoregulatory site in the promoter of *Tbx4*. However, unknown diffusible factor(s) induced by *Tbx5* might also suppress *Tbx4* expression in the leg bud.

Figure 5 summarizes the regulatory interactions of genes that specify the wing/leg identity of limb buds. Although we are far from a complete understanding of how the morphologies of the vertebrate limbs diverged during evolution^{1,2,29} and whether the structural difference of the *Tbx5* and *Tbx4* proteins might explain the morphological variations in vertebrate limbs, further analysis of the vertebrate *Tbx5/Tbx4* genes will be essential to answer these equations. □

Methods

Cloning of *Tbx5/Tbx4* and *Pitx1* genes. A chick limb-bud complementary DNA library prepared from poly(A)⁺ RNA isolated from stage 20–23 limb bud

was screened with a human *TBX2* cDNA probe (a gift from C. E. Campbell). Positive clones were subjected to sequencing analysis (ABI sequencer). After searching the data base, four positive clones were identified as chick *Tbx5* and *Tbx4*. The *Pitx1* clone was obtained by screening the same library with the mouse *Crx* gene as a probe.

Whole-mount *in situ* hybridization and skeletal pattern analysis. Whole-mount *in situ* hybridization was performed as described³⁰. *Hoxc9* and *Hoxd9* probes have been described^{6–8}. To stain skeletal elements, embryos were fixed and stained with Alcian blue¹⁶.

RCAS retrovirus DNA and electroporation. The *Tbx5* and *Tbx4* coding regions were PCR amplified, then ligated to a pSLAX vector²³. For GFP fusion constructs, the *Tbx5* and *Tbx4* coding regions were PCR-amplified with primers designed to fit the open reading frame of pEGFP-N2 (Clontech). The resultant *Tbx5*– and *Tbx4*–EGFP fusion cDNAs were ligated to a pSLAX vector. pSLAX–*Tbx5*–EGFP and pSLAX–*Tbx4*–EGFP were restricted with *Cla*I and the fusion DNA fragments were ligated to the *Cla*I site of a pRCASBP retrovirus vector²³. For electroporation²², a BTX T-820 electroporator (BTX) and a pulse monitor (Meiwa Shoji) were used. A platinum electrode (Muramatsu) and a sharpened tungsten needle (Nilaco) were used as an anode and a cathode, respectively. Fertilized eggs were purchased from Takeuchi poultry farm (Nara). A small window was opened for access. PBS(–) was poured over the embryo to obtain an appropriate resistance (<0.5 Ω). An anode and a cathode were laid down near the posterior and anterior portions of the embryo, respectively. While DNA solution was being injected into the mesenchymal field with a sharp glass pipette, electric pulses were applied (12–16 V, 90 ms, 1–3 times). After sealing the egg-shells, embryos were allowed to develop in humidified incubators.

Cell implantation. Chicken embryonic fibroblast (CEF) cells and wing mesenchyme cells were transfected with RCASBP-chick FGF8 and RCASBP–*Tbx4*–EGFP retrovirus plasmids, respectively, by the calcium phosphate method. Transfected cells were cultured to allow the retrovirus to spread. Then FGF8-producing CEF cells and *Tbx4*-expressing mesenchyme cells were mixed, centrifuged briefly and incubated for at least 2 h at 37 °C to make tight aggregates. Cell aggregates were implanted into the flanks at the levels of somite 21 at stage 14–15.

Received 5 February; accepted 24 March 1999.

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Acknowledgements. We thank C. Campbell for human *TBX2* cDNA plasmid pQE9Eag; J. C. Izpisua-Belmonte for chicken *Hoxd9*; A. Kuroiwa for chicken *Hoxb9* and *c9* probes; K. Umesono and R. Yu for critically reading the manuscript and for helpful comments; and T. Momose for technical assistance in the electroporation system. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, and by special coordination funds for promoting science and technology from the Science and Technology Agency. A.V.-H. was supported by a grant from the Deutsche Forschungsgemeinschaft and a foreign fellowship from the Japan Society for the Promotion of Sciences. This paper is dedicated to K. Umesono.

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The T-box genes *Tbx4* and *Tbx5* regulate limb outgrowth and identity

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During embryonic development, initially similar fields can develop into distinct structures, such as the vertebrate fore- and hindlimbs. Although considerable progress has been made in our understanding of the genetic control underlying the establishment of the different limb axes^{1–3}, the molecular cues that specify the differential development of the fore- and hindlimbs are unknown. Possible candidates for genes determining limb identity are *Pitx1*, a gene whose transcripts are detected in the early hind- but not forelimb bud, and two members of the T-box (*Tbx*) gene family, *Tbx4* and *Tbx5*, which are specifically expressed in the hindlimb and forelimb buds, respectively^{4–6}. Here we show that *Tbx4* and *Tbx5* are essential regulators of limb outgrowth whose roles seem to be tightly linked to the activity of three signalling proteins that are required for limb outgrowth and patterning: fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and Wnt. In addition, we provide evidence that *Tbx4* and *Tbx5* are involved in controlling limb identity. Our findings provide insight into how similar developmental fields can evolve into homologous but distinct structures.

In chick embryos, leg grafts expressing *Tbx4* transcripts can develop into toe-like digits when placed into the wing. Conversely, wing grafts expressing *Tbx5* transcripts can develop into wing-like digits when placed into the leg^{7–10}. Thus, if *Tbx5* and *4* are involved in specifying limb identity, their ectopic expression could induce leg cells to become wing cells and vice versa. This was initially investigated by injecting replication-competent retroviral vectors (RCAS), or replication deficient adenoviral vectors (Ad), containing chick *Tbx4* and *Tbx5* complementary DNAs into presumptive chick leg (*Tbx5*) and wing (*Tbx4*) cells between developmental stages 8 and 12 (ref. 11).

The most common phenotype after viral injection had arrested limb development (Fig. 1). Most of the heavily infected limb buds were smaller than the contralateral non-infected limbs and showed changes in the apical ectodermal ridge (AER); the specialized

epithelium located at the distal tip of the limb bud which is required for normal limb outgrowth¹²) (Fig. 1a–d). These alterations included the lack of a defined pseudostratified epithelium, as well as disruption and splitting of the AER into two or more domains. We saw such changes in 73% of *Tbx4*-injected wings and 48% of *Tbx5*-injected legs. Limb elements were truncated in infected embryos at later stages of development. The most common phenotypes were the hypoplasia or disappearance of zeugopodal elements, typically the radius in the wing (Fig. 1f) and the fibula in the leg (Fig. 1i, j), and the absence of anterior digits (digit II in the wing (Fig. 1f) and digits I and II in the leg (Fig. 1l). The alterations observed after *Tbx4* misexpression resembled the phenotypes described in human mutations for *Tbx5* which include abnormalities of the most anterior hand digits, and hypoplasia and/or absence of the radius¹³. These results suggested that, in our experimental setup, ectopic expression of *Tbx4* could downregulate the activity of *Tbx5* and vice versa.

The T-box domain binds DNA as a dimer¹⁴. The observed alterations may be due to the formation of heterodimers between the ectopic and endogenous proteins, which could inactivate the endogenous protein. This, in turn, would prevent the activation of downstream target genes which may include the *Tbx* genes themselves. Ectopic expression of *Tbx5* in the leg bud can downregulate *Tbx4* messenger RNA (Fig. 2a) as well as that of *Tbx2* and *Tbx3*, two other T-box genes expressed in both the fore- and hindlimb buds (data not shown). Overexpression of *Tbx4* in the leg bud also induced limb truncations (which were also preceded by downregulation of *Tbx2* and *Tbx3*; data not shown). Our results therefore indicate that the alterations in limb outgrowth observed after *Tbx5*/4 misexpression, rather than reflecting a qualitative change, might reflect a change in the total amount of Tbx proteins present during limb outgrowth.

The transcription of genes involved in limb outgrowth, such as *Bmp2* (refs 15–19), was reduced. Mesenchymal *Bmp2* transcripts were greatly diminished 60 h after viral injection with either *Tbx4* (RCAS; Fig. 2b) or *Tbx5* (adenovirus; data not shown). Other genes implicated in limb outgrowth include members of the FGF family^{1–3}. Ectopic expression of *Tbx5* and *Tbx4* downregulated the expression of *Fgf4*, -8 and -10 (Fig. 2c–e). Downregulation of transcripts of *Wnt3a*, a gene that induces FGF signalling and hence limb outgrowth²⁰, was also observed 48–60 h after viral infection (Fig. 2f). Hence, the perturbations in limb outgrowth after ectopic expression of *Tbx4* and *Tbx5* may be due to altered gene activity in three of the known pathways implicated in vertebrate limb outgrowth: BMP, WNT and FGF.

To determine whether the expression of *Tbx4* and *Tbx5* in the chick limb bud is, in turn, regulated by the WNT, BMP and FGF signalling pathways, we performed gain- and loss-of-function experiments with members of these pathways that are essential for normal limb outgrowth. Although *Tbx5* transcripts are specifically restricted to the forelimb bud in the initial stages of limb-bud outgrowth, at stage 24 they are also found in a few cells located in the dorsal side of the leg bud (Fig. 2g). Beads soaked in *Bmp2* and implanted in stage-20 leg buds induced the ectopic appearance of *Tbx5* transcripts (Fig. 2h). The complementary experiment, (blocking BMP signalling by infecting presumptive limb cells at stage 12 with a dominant-negative BMP receptor^{16–18}) caused *Tbx5* transcripts to disappear from the dorsal side of the leg bud (Fig. 2i), as well as to be downregulated in the wing bud (data not shown). Ectopic expression of *Wnt3a* caused a similar downregulation (Fig. 2j).

Because the FGF signalling pathway is also essential for vertebrate limb development, we investigated whether *Tbx4* and *Tbx5* are controlled by this pathway. FGF beads can regulate *Tbx* expression in the flank^{7–10}, and upregulate *Tbx5* expression (Fig. 2k) when implanted in the leg bud at stage 18. Infection of presumptive limb cells at stages 8–12 with a retroviral vector containing the domi-

nant-negative Raf-1 mutant NAF (which blocks FGF action in *Xenopus*)²¹ caused arrested limb-bud outgrowth (Fig. 2l, n). Transcripts for both *Tbx5* (Fig. 2m) and *Tbx4* (not shown) mRNA were downregulated 48–60 h after infection. Overexpression of a dominant-negative *Fgfr2* construct showed similar changes (data not shown). Further evidence for the ability of FGF signalling to control *Tbx5/4* gene transcription is seen in mice lacking *Fgf10* (ref. 22) or *Fgfr2* (C. Deng, NIH, Bethesda, personal communication), which show downregulation of *Tbx4* and *Tbx5*. Although the precise mechanism by which FGF signalling regulates *Tbx5/4* expression during limb development is unknown, the results shown in Fig. 2e and k–n agree with mechanisms occurring during *Xenopus* development, where *Brachyury* (another *Tbox* gene) and *Fgf* are components of a regulatory loop in which each maintains expression of the other (see ref. 23 and references therein). Our results indicate that there may be a feedback loop between *Tbx5* and *Tbx4* and members of the FGF, BMP and WNT pathways, and identify *Tbx5/4* as essential regulators for organizing the proximodistal outgrowth of the chick limb. These findings may provide insight into the molecular processes underlying the aetiology of human syndromes in which *Tbx* function is altered.

The above results indicate that the *Tbx5/4* genes are critical for promoting limb outgrowth. They do not, however, implicate these genes in establishing and/or maintaining wing/leg cell-fate determination. By the time the *Tbx5/4* viruses are active, limb identity may already have been determined, so that limb transdetermination could not occur. Moreover, transdetermination is less likely to occur in the absence of cell proliferation²⁴. Hence, it might not be possible to determine the putative role of *Tbx5* and *Tbx4* in establishing limb identity in our experimental setup, where cell proliferation is arrested owing to alterations in the activity of some members of the BMP, WNT and FGF signalling pathways.

If FGF is applied to the prospective flank, an ectopic limb

develops. The identity of this limb correlates with the position where the FGF bead was applied. Beads positioned close to the wing bud (somite 21) induce a wing, whereas beads close to the leg bud (somite 25) induce a leg²⁵. To determine whether anterior flank cells (when induced to proliferate and form a wing after being exposed to FGF) can also form a leg, we induced ubiquitous expression of *Tbx4* in the flank by injecting *Tbx4* RCAS in the presumptive flank cells at stage 8. Later, at stage 15, beads soaked in FGF were implanted in anterior flank cells (somite 21) of *Tbx4* RCAS-infected embryos. Application of FGF beads opposite somite 21 induces *Tbx5* expression throughout (Fig. 3a), and *Tbx4* expression in the most posterior part, but not in the anterior (Fig. 3b), of the ectopic limb bud^{7–10}. However, the combined expression of FGF beads and *Tbx4* RCAS downregulated *Tbx5* expression (Fig. 3c) and induced *Tbx4* throughout the ectopic limb bud (Fig. 3d), indicating that *Tbx4*, like *Brachyury* in *Xenopus*²³, can induce its own transcription. To determine whether *Tbx4* misexpression alters the expression of hindlimb-specific markers, we used *in situ* hybridization for *Hoxc11*, whose transcripts are specifically found in the leg but not the wing bud. Ectopic expression of both FGF and *Tbx4* in the flank induced *Hoxc11* (Fig. 3f), whereas FGF and *Tbx4* did not induce *Hoxc11* in the wing (Fig. 3e). When the ectopic limbs were allowed to develop further, three major phenotypes were obtained: 54% of the ectopic limbs had a typical leg morphology (Fig. 3i, j) (in control experiments, where the application of FGF was not preceded by *Tbx4* RCAS infection, 21% were legs); 26% were wings (in control experiments, 68% were wings; Fig. 3g, h); and 20% had an indeterminate morphology (11% in control experiments). A few embryos had truncated wings similar to those seen after ectopic *Tbx4* expression in the wing. These results indicate that the presence of *Tbx4* in the anterior flank mesoderm may be able to transdetermine flank cells from wing to leg fate when the flank is transformed into limb territory by FGF.

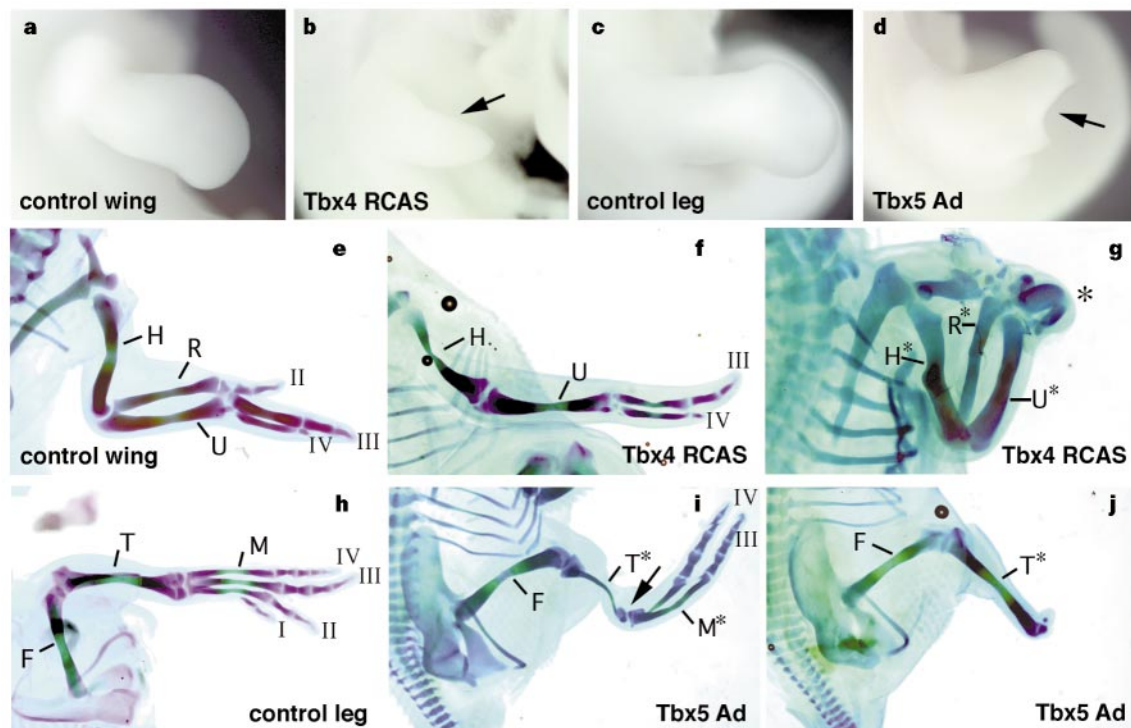


Figure 1 *Tbx5* and *Tbx4* regulate limb outgrowth. **a, c**, Wild-type chick limb buds. **b, d**, Injection of a retroviral vector containing *Tbx4* cDNA (**b**) or adenovirus containing *Tbx5* cDNA (**d**) in presumptive wing and leg cells, respectively, at stages 8–12 cause deletions of distal limb tissue and perturbation of the AER (black arrows in **b** and **d**). **e–j**, Wild-type (**e, h**), *Tbx4*- (**f, g**) and *Tbx5*- (**i, j**) injected limbs 8 days after infection at stages 8–12. Truncations occur mainly in the

zeugopodal and autopodal segments (**f, i**) where the radius and fibula are either absent or reduced in length and the most anterior digits (digit II in the wing; digits I and II in the leg) are absent. In more severe cases, the infected limbs lacked the entire distal part of the limb (**g, j**). H, humerus; R, radius; U, ulna; F, femur; T, tibiotarsus; M, metatarsus; roman numerals denote digit identity; asterisks indicate affected elements.

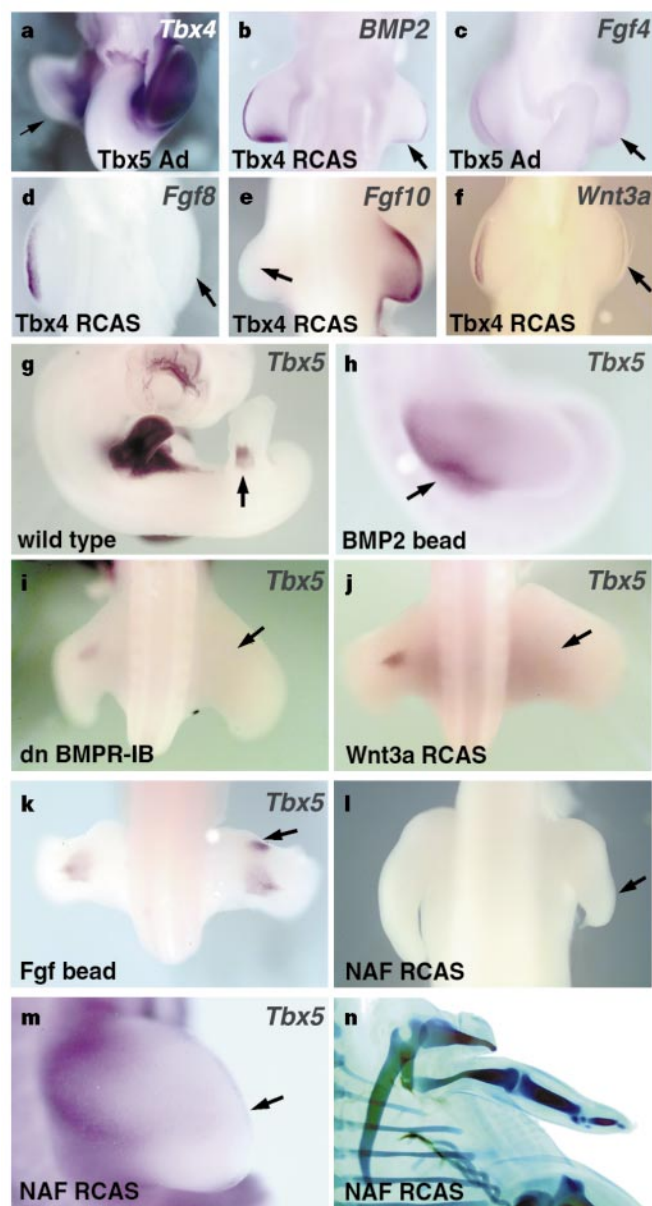


Figure 2 Feedback regulatory loops between *Tbx5/4* and the FGF, WNT and BMP signalling pathways. *In situ* probes are indicated in italics and the misexpressed gene in black. **a**, *Tbx4* transcripts are downregulated at the distal leg bud (arrow) after adenoviral misexpression of *Tbx5*. **b**, **d**–**f**, Retroviral infection of *Tbx4* causes downregulation (black arrow) of *Bmp2* (**b**), *Fgf8* (**d**), *Fgf10* (**e**), and *Wnt3a* (**f**). **c**, *Fgf4* transcripts are almost absent (arrow) after adenoviral infection of *Tbx5*. We tried to restrict *Tbx4* infection to the mesoderm, but it is almost impossible not to infect the ectoderm as well. Hence, it is not known whether gene downregulation occurs primarily in the mesoderm and indirectly in the AER or vice versa. The fact that mesodermal transcripts were lost more frequently than ectodermal transcripts indicates that downregulation of gene expression may occur initially in the mesoderm and subsequently in the AER. **g**, Wild-type expression of *Tbx5*. *Tbx5* transcripts are detected in a small population of dorsal mesenchymal leg bud cells (arrow) as well as throughout the forelimb bud. **h**, BMP2 beads applied at the dorsal side of stage 19 leg buds induce *Tbx5* transcripts (arrow). **i**, **j**, Blocking of BMP signalling with a dominant-negative BMP receptor (**i**) or overexpression of *Wnt3a* (**j**) abolish leg bud *Tbx5* expression (arrows). **k**, In contrast, *Tbx5* transcripts are upregulated (arrow) after implantation of an FGF2 bead at the dorsal side of stage 18 leg buds. **l**, **n**, Blocking FGF signalling using a retroviral vector containing a dominant-negative form of Raf1 arrests limb outgrowth (arrow in **l**; compared with contralateral non-injected limb bud). *Tbx5* transcripts are downregulated at the distal part of NAF-RCAS injected limb buds (arrow). After further development, infected limb buds had missing limb elements, including the radius and two digits.

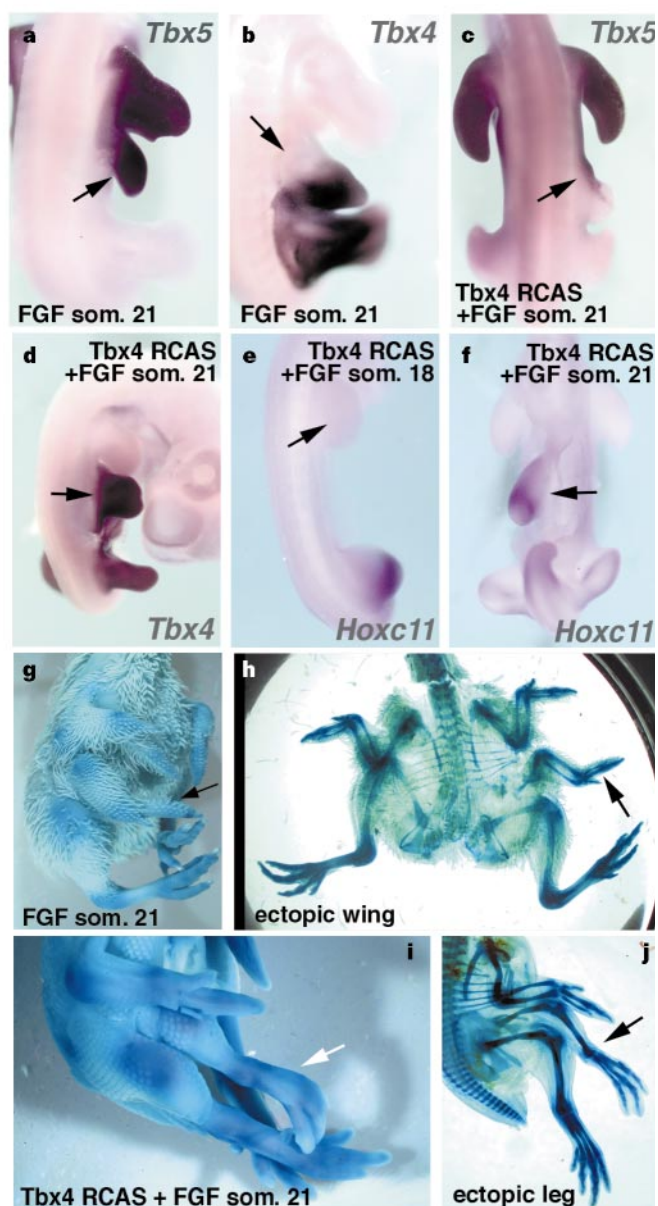


Figure 3 FGF and *Tbx4* change the fate of anterior flank cells from wing to leg. Key is as in Fig. 2. **a**, **b**, Application of FGF2 beads opposite somite 21 induces *Tbx5* expression throughout most cells of ectopic limb bud (arrow in **a**). However, *Tbx4* transcripts are induced at the posterior but not the anterior site of ectopic limb bud (arrow in **b**). **c**, **d**, Combined expression of FGF2 and *Tbx4* alters the previous pattern of expression, reducing *Tbx5* transcripts (arrow in **c**) and inducing *Tbx4* transcripts throughout the entire ectopic limb bud (arrow in **d**). This pattern resembles the pattern of *Tbx5/4* expression that correlates with the development of leg but not forelimb structures. **e**, **f**, *Hoxc11* transcripts, normally confined to leg buds, are induced in ectopic limb buds (arrow in **f**) that develop after application of FGF2 beads opposite somite 21 of embryos previously infected with *Tbx4* RCAS. When FGF beads were applied opposite somite 18 of previously *Tbx4*-RCAS-infected embryos, no upregulation of *Hoxc11* was detected (**e**). **g**–**j**, Implantation of FGF beads opposite somite 21 gives rise to an ectopic wing (**g**, **h**, arrows, 70% of cases) and ectopic legs (20% of cases). However, if the presumptive anterior flank cells are injected with the *Tbx4* RCAS construct before FGF bead implantation, the percentage of legs produced increases to 54% (**i**, **j**, arrows) and the percentage of wings decreases to 26%.

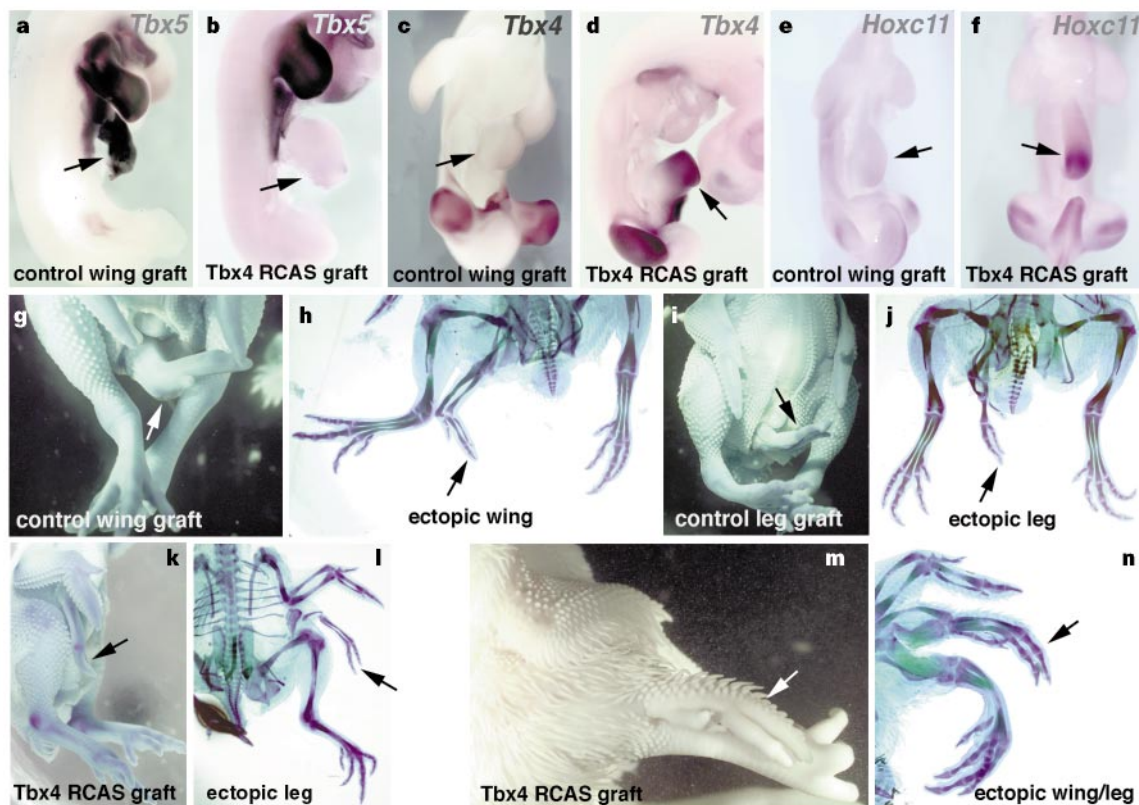


Figure 4 *Tbx4* promotes 'legness'. Key is as in Fig. 2. **a, c, e**, Grafting of stage 13 presumptive wild-type wing cells to the coelom of stage 15–16 host embryos results in expression of *Tbx5* throughout the ectopic limb (arrow in **a**). No *Tbx4* (**c**) and *Hoxc11* (**e**) transcripts are seen (arrows). **g, h**, When operated embryos are allowed to develop further, they generate ectopic wing elements (arrows). Grafting of *Tbx4*-RCAS-infected presumptive wing tissue can prevent, in some cases, the appearance of *Tbx5* transcripts (**b**, arrow) and induce *Tbx4* (**d**) and

Hoxc11 (**f**) transcripts in the induced limb buds. **k, l**, Direct whole-mount observation and cartilage staining of the embryos that had developed further showed leg elements (arrows) very similar to those seen after grafting wild-type non-*Tbx4*-RCAS-infected presumptive leg explants (**i, j**, arrows). In other cases, chimaeric limbs composed of wing (feathers in **m** and a small digit in **n**) and leg (two large digits in **m** and **n**) elements are observed after grafting *Tbx4*-RCAS-infected presumptive wing tissue.

Our findings indicate that, once the genetic regulatory interactions that determine 'wingness' are set in train in the wing field, a constraint mechanism prevents the adoption of the 'legness' program. We tested whether forcing *Tbx4* expression in limb field cells before limb identity is determined²⁶, and placing them away from the influence of presumptive wing cells, could induce 'legness'. Stage-4–6 chick embryos were injected with *Tbx4* RCAS in the lateral plate mesoderm. Explants of *Tbx4*-infected presumptive wing tissue were excised at stage 13 (chick limb identity is already determined at stage 12) and grafted, not in the flank, where they would be exposed to 'wingness' cues, but into the coelom of 2.5–3-day-old chick embryos. *In situ* hybridization (using a 3' untranslated *Tbx4* probe not included in the *Tbx4* RCAS construct) at 48–60 h after grafting detected *Tbx4* transcripts (three out of four embryos) in the limb-bud-like tissue that protruded from the abdominal cavity (Fig. 4d). In contrast, *Tbx5* transcripts, which are always present throughout the ectopic limb bud after grafting non-infected *Tbx4* tissue (Fig. 4a), were either not present (two out of five embryos; Fig. 4b), or downregulated (three out of five embryos; data not shown). As after the misexpression of FGF and *Tbx4* in the flank, we also detected transcripts for *Hoxc11* (Fig. 4f). Grafts of non-*Tbx4*-infected tissue never induced *Tbx4* (Fig. 4c) or *Hoxc11* expression (Fig. 4e). Skeletal analysis of the infected embryos that were left to develop further revealed the presence of ectopic leg elements (Fig. 4k, l), or chimaeras composed of both wing and leg elements (Fig. 4m, n). Non-infected wing (Fig. 4g, h) or leg (Fig. 4i, j) explants produced only wing or leg elements, respectively. These results indicate that *Tbx4*, if present in the presumptive wing cells before their identity is fully determined,

can initiate a pathway of differentiation that leads to 'legness'.

Comparative loss-of-function, biochemical and evolutionary studies are still needed to determine the regulatory genetic cascade that initiates the differences in gene expression that transform two similar developmental fields into two homologous but distinct structures. However, when combined with previous observations in both vertebrates^{4–5,27} and invertebrates (refs 28, 29 and references therein), our findings will allow us to start deciphering how the initial cues present in the limb field are translated into the morphological differences that distinguish the vertebrate fore- and hindlimb. □

Methods

The *in situ* hybridization protocol, mRNA probes, cartilage-staining procedure, retroviral and adenoviral preparation, and infection protocols have been described^{8,20,30}. The dominant BMP receptor and NAF constructs are described in refs 17 and 21, respectively. Grafting procedures were done as described²⁶. Detailed protocols are available upon request from the authors.

Received 8 February; accepted 5 March 1999.

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Acknowledgements. We thank A. Ryan and Y. Kanegae for help in the initial phase of this work; D. Büscher, J. Capdevila, J. K. Ng and A. T. Tavares for comments; C. Tabin, S. Simonet and C. Deng for unpublished results; C. Tabin, Y. Kawakami and L. T. Williams for reagents; and L. Hooks for help in preparing the manuscript. This work was supported by grants from the NSF and the G. Harold and Leila Y. Mathers Foundation to J.C.I.B., who is a Pew Scholar.

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Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus

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In eukaryotes, the activation of mitotic cyclin-dependent kinases (CDKs) induces mitosis, and their inactivation causes cells to leave mitosis¹. In budding yeast, two redundant mechanisms induce the inactivation of mitotic CDKs. In one mechanism, a specialized ubiquitin-dependent proteolytic system (called the APC-dependent proteolysis machinery) degrades the mitotic (Clb) cyclin subunit. In the other, the kinase-inhibitor Sic1 binds to mitotic CDKs and inhibits their kinase activity^{1,2}. The highly conserved protein phosphatase Cdc14 promotes both Clb degradation and Sic1 accumulation. Cdc14 promotes *SIC1* transcription and the stabilization of Sic1 protein by dephosphorylating Sic1 and its transcription factor Swi5. Cdc14 activates the degradation of Clb cyclins by dephosphorylating the APC-specificity factor Cdh1 (refs 3, 4). So how is Cdc14 regulated? Here we show that Cdc14 is sequestered in the nucleolus for most of the cell

cycle. During nuclear division, Cdc14 is released from the nucleolus, allowing it to reach its targets. A highly conserved signalling cascade, critical for the exit from mitosis, is required for this movement of Cdc14 during anaphase. Furthermore, we have identified a negative regulator of Cdc14, Cfi1, that anchors Cdc14 in the nucleolus.

Overexpression of the *CDC14* gene in yeast induces ectopic inactivation of mitotic kinases³, indicating that the Cdc14 protein is a limiting factor in promoting exit from mitosis that needs to be tightly regulated. Amounts of Cdc14 do not fluctuate during the cell cycle (results not shown), but the subcellular localization of Cdc14 changes markedly. To demonstrate this, we tagged the chromosomal copy of *CDC14* with a haemagglutinin epitope (Cdc14–HA; ref. 5) and visualized Cdc14–HA by indirect *in situ* immunofluorescence. During G1, S phase and early mitosis, Cdc14–HA was localized to granules in a crescent shape in the nucleus (Fig. 1b–d). During mitosis, Cdc14–HA spread to the nucleus and, to some extent, to the cytoplasm (Fig. 1b, arrowhead). Analysis of Cdc14–HA localization in a synchronous culture indicated that Cdc14–HA was released from the nucleolus during anaphase (Fig. 1e). The movement of Cdc14–HA during anaphase takes place when Cdc14 activity is required to dephosphorylate its targets, Sic1, Cdh1 and Swi5 (refs 3, 4).

The subnuclear localization of Cdc14–HA was similar to that of nucleolar proteins. Cdc14–HA co-localized with the nucleolar proteins Nop1 (ref. 6) and Sir2 (ref. 7; Fig. 1f, and data not shown), but not with nuclear proteins such as the predominantly telomeric protein Rap1 (ref. 8), the DNA synthesis initiation factor complex Orc (ref. 9) and the sister-chromatid cohesion factor Scc1/Mcd1 (refs 10, 11; data not shown).

A poorly understood signalling cascade, the ‘mitotic exit pathway’, promotes the inactivation of mitotic kinases at the end of mitosis. Components of this signalling cascade include the protein kinases Cdc15 (ref. 12), Cdc5 (ref. 13), Dbf2, Dbf20 (ref. 14), the GTP-binding protein Tem1 (ref. 15) and the guanine-nucleotide-exchange factor Lte1 (ref. 16). Overexpression of Cdc14 bypasses the requirement of Cdc5, Dbf2 and Cdc15 for the degradation of Clb cyclins and accumulation of Sic1 (suppression of *tem1* and *lte1* mutants had not been tested), indicating that Cdc14 is the ultimate effector of the mitotic exit pathway³. Cdc5, Cdc15, Dbf2, Dbf20 and Tem1 are all required to release Cdc14–HA from the nucleolus during anaphase. Cells carrying a temperature-sensitive allele in any of these genes arrest in telophase with Cdc14–HA remaining in the nucleolus (Fig. 2a, b, and data not shown). Cells overexpressing a non-degradable form of Clb2 (Clb2dBA) also arrest in telophase¹². Release of Cdc14–HA from the nucleolus was not affected by overexpression of the *CLB2dBA* mutant (Fig. 2c). This is consistent with previous findings showing that Cdc14 functions upstream of Clb2 destruction during exit from mitosis³. The findings that Cdc14 spreads throughout the cell when its function is required to trigger exit from mitosis, that Cdc14 fails to spread to the nucleus and cytoplasm in cells mutated for genes important for Cdc14 function, and that overexpressed Cdc14–HA is present in the nucleus, and to some extent in the cytoplasm, throughout the cell cycle (Fig. 2d) and causes ectopic degradation of mitotic cyclins and hyper-accumulation of Sic1 (ref. 3), all indicate that release of Cdc14 from the nucleolus must be a critical step in Cdc14 activation during anaphase.

To determine whether sister-chromatid separation itself triggers release of Cdc14–HA from the nucleolus during anaphase, we analysed the localization of Cdc14–HA in cells that either fail to separate sister chromatids (*esp1-1* mutant; ref. 17) or cells that separate sister chromatids prematurely (*scc1/mcd1* mutant; refs 10, 11). *esp1-1* mutants progress through the cell cycle normally until metaphase, when they disassemble the metaphase spindle and exit mitosis without having undergone nuclear division and sister-chromatid separation^{12,17,18}. Figure 2e (arrowhead) shows that,

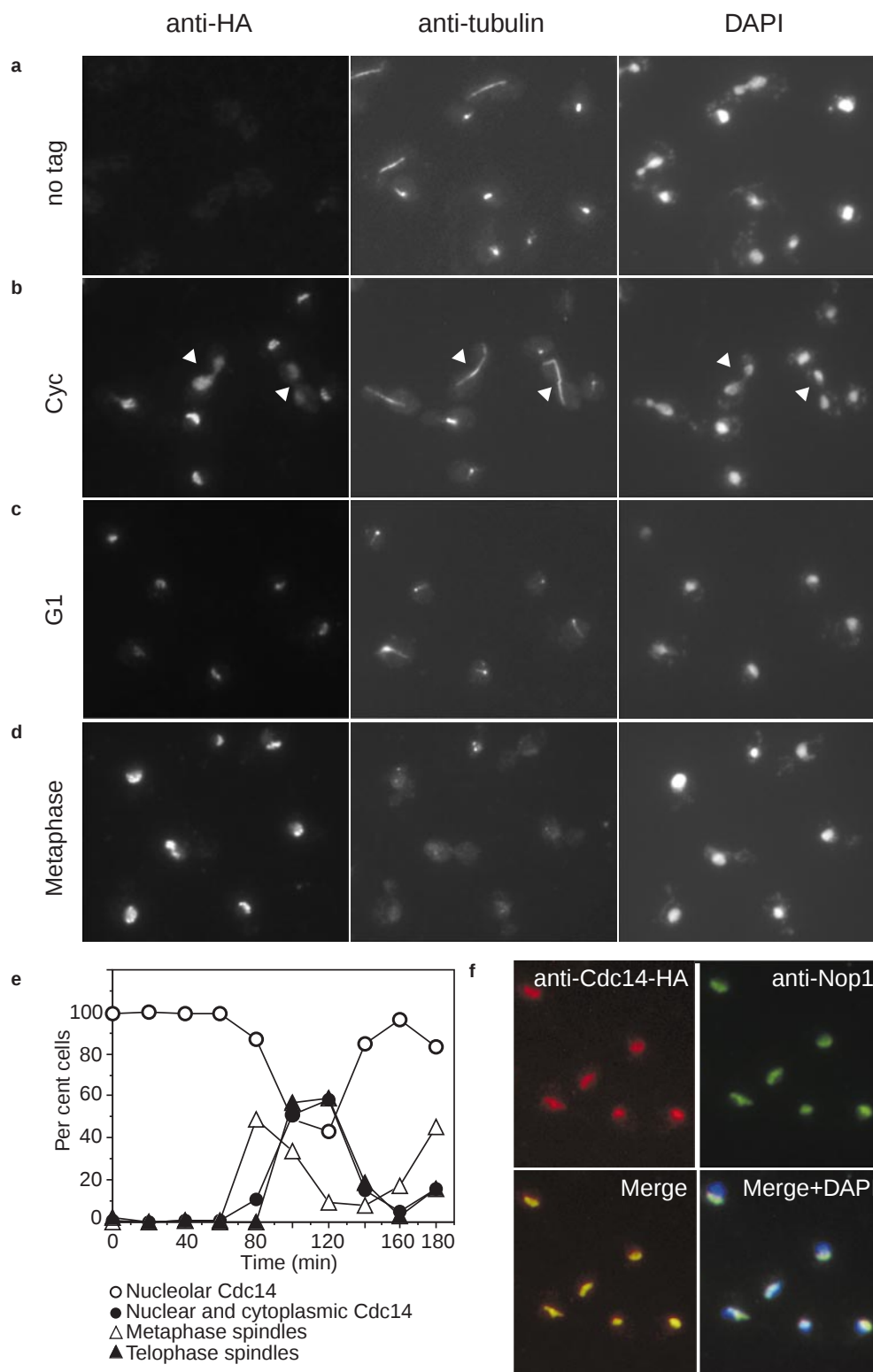


Figure 1 Subcellular localization of Cdc14-HA during the cell cycle. **a**, Exponential growing wild-type cells (K699) without a *CDC14-HA* fusion (no tag). **b-d**, Exponentially growing (**b**), G1 (using α -factor pheromone; **c**) and metaphase (using the microtubule depolymerizing drug nocodazole; **d**)-arrested Cdc14-HA cells (A1411). Anti-HA, staining using anti-HA antibodies; anti-tubulin, staining using anti-tubulin antibodies²⁷; DAPI (4',6-diamidino-2-phenylindole) was used to

stain DNA. Arrowheads indicate cells in telophase. **e**, Synchronous culture after release from an α -factor-induced G1 arrest showing the percentage of cells with Cdc14-HA in the nucleolus, with Cdc14-HA in the nucleus and cytoplasm, and with metaphase and telophase spindles. **f**, Co-localization of Cdc14-HA with Nop1 in G1 cells.

despite the failure of *esp1-1* mutants to separate sister chromatids and to undergo nuclear division, Cdc14-HA dissociates from the nucleolus. Cells defective in the cohesin *Scc1/Mcd1* show premature sister-chromatid separation when arrested in metaphase using the

microtubule-depolymerizing drug nocodazole^{10,11}. Cdc14-HA was not released from the nucleolus under these conditions (Fig. 2f). These findings indicate that sister-chromatid separation itself does not trigger the release of Cdc14 from the nucleolus, but rather that

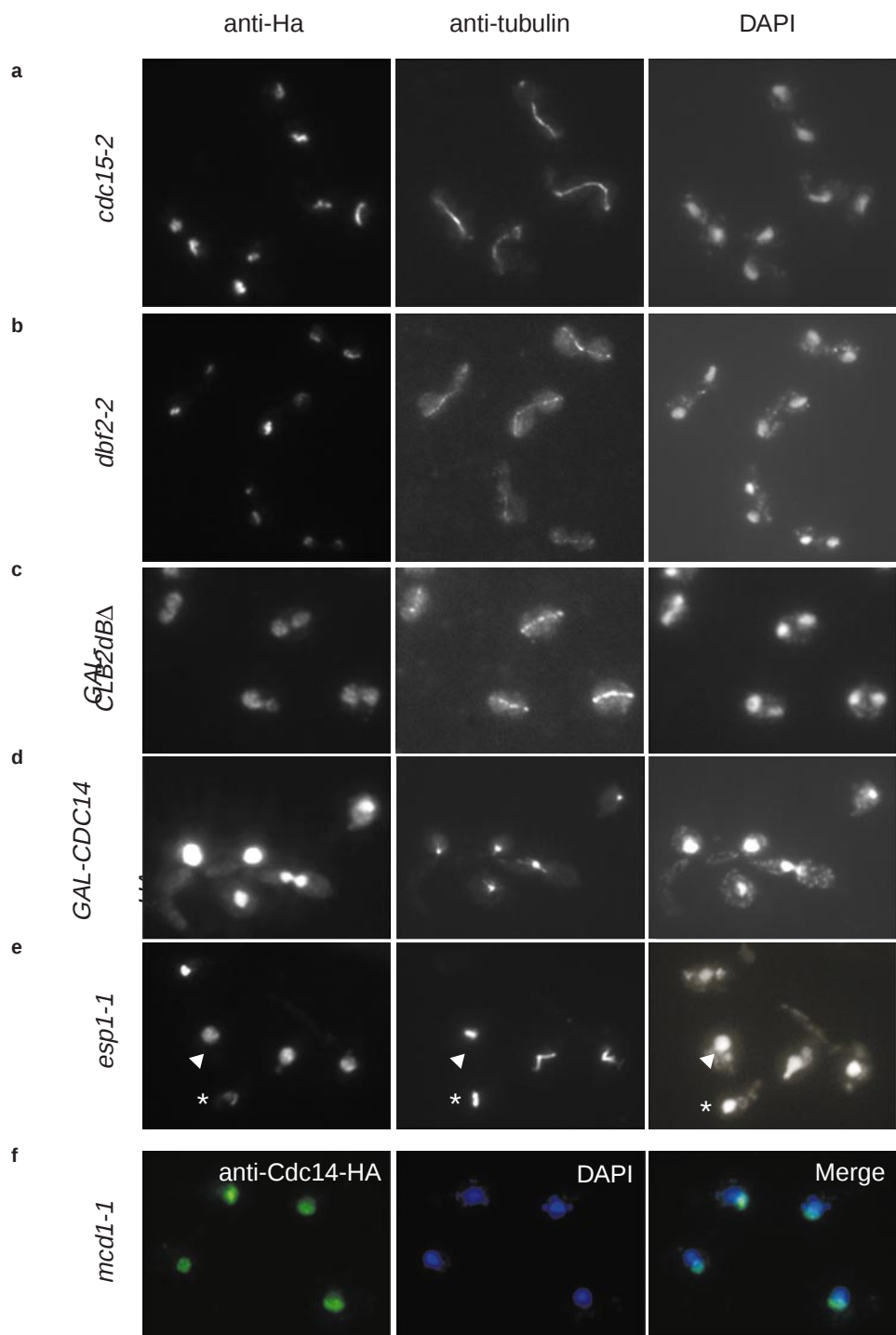


Figure 2 Cdc14 localization in various cell cycle mutants. **a, b**, Cdc14-HA in *cdc15-2* (**a**; A1674) or *dbf2-2* mutants (**b**; A1676) arrested at 37 °C for 2.5 h. **c, d**, Cdc14-HA in cells overexpressing *Clb2dBA* (**c**; A1731) or *CDC14-HA* (**d**; A1367) from the *GAL1-10* promoter. **e**, Cdc14-HA in *esp1-1* cells (A1730) 80 min after

release at 37 °C from an α -factor-induced G1 arrest. Asterisk: Cdc14-HA in the nucleolus; arrowhead: Cdc14-HA in the nucleus. **f**, Cdc14-HA in *mcd1-1* cells (A1724) arrested with nocodazole, followed by shift to 37 °C for 45 min to induce sister-chromatid separation.

regulators of this process, such as Cdc20 (ref. 19), do. This would be consistent with the previous finding that *CDC20* also functions during exit from mitosis^{20,21}.

To find out how Cdc14 localization is regulated, we used a yeast 2-hybrid screen²² to identify potential Cdc14-interacting proteins. This screen identified open reading frame YJL076w as a potential Cdc14 interactor, which we termed *CFI1* (Cdc14 inhibitor 1). The Cfi1 protein shows weak homology (27% similarity over 470 amino acids) to Reg1, a regulatory subunit for protein phosphatase 1 (ref. 23). Two regions shared a higher degree of homology. In region 1

(Fig. 3a, top) Cfi1 and Reg1 were 33% identical and 45% similar; in region 2 (Fig. 3a, bottom) Cfi1 and Reg1 were 29% identical and 39% similar.

Cdc14 and Cfi1 interacted not only in the 2-hybrid assay but also in yeast *in vivo*. The chromosomal Cfi1 tagged with a Myc epitope immunoprecipitated the chromosomal Cdc14 tagged with a haemagglutinin epitope (Fig. 3b). Cfi1-Myc and Cdc14-HA also co-localized in the nucleolus (Fig. 3c). Thus, Cdc14 and Cfi1 interact, as judged by 2-hybrid, co-immunoprecipitation and co-localization analyses. However, whereas Cdc14 spreads throughout

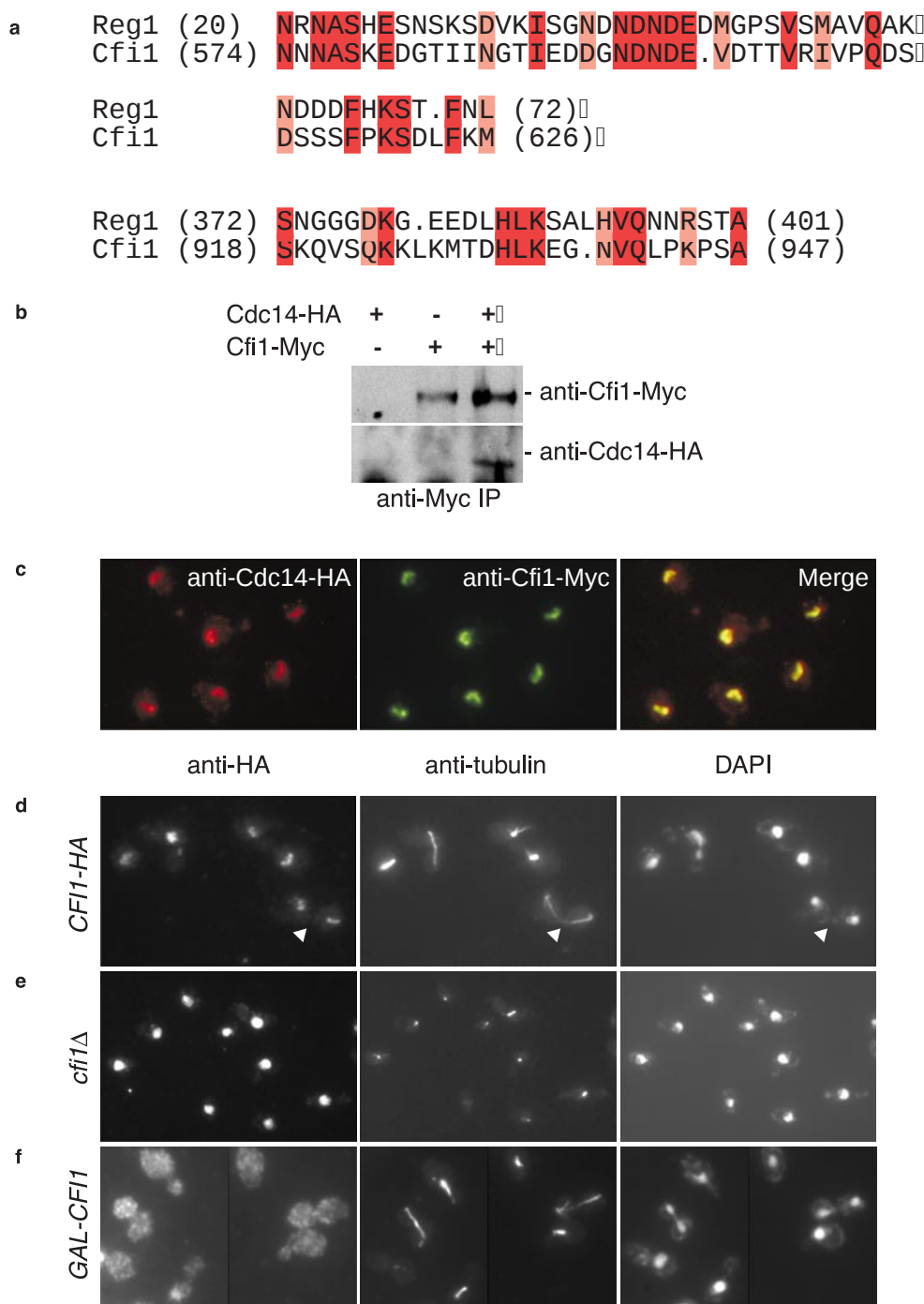


Figure 3 Cfi1 interacts with Cdc14 and regulates its subcellular localization. **a**, Sequence alignment of Cfi1 and Reg1. Identities are boxed in red, similarities in pink. **b**, Cfi1-Myc was immunoprecipitated from exponentially growing Cdc14-HA (A1411), Cfi1-Myc (A1638) or Cdc14-HA, Cfi1-Myc cells (A1681). Immunoprecipitates were probed for the presence of Cdc14-HA by immunoblot analysis.

c, Co-localization of Cfi1-Myc and Cdc14-HA (A1681) in the nucleolus in G1 cells. **d**, Localization of Cfi1-HA (A1639). Arrowhead indicates cell in telophase. **e**, **f**, Localization of Cdc14-HA in cells lacking *CFI1* (**e**; A1690) and cells overexpressing *CFI1* (**f**; A1700).

the cell during anaphase, Cfi1 remains in the nucleolus (Fig. 3d, arrowhead).

In cells lacking *CFI1*, Cdc14-HA was localized in the nucleus, and to some extent in the cytoplasm, throughout the cell cycle (Fig. 3e). In contrast, deletion of another nucleolar protein-encoding gene, *SIR2*, did not affect the nucleolar localization of Cdc14-HA (data not shown). Overexpression of *CFI1* causes the Cfi1 protein to be present throughout the cell (data not shown). In such cells, Cdc14-HA was no longer sequestered in the nucleolus but was also present throughout the cell (Fig. 3f), showing that mislocalization of Cfi1

leads to mislocalization of Cdc14-HA. Thus, Cfi1 is responsible for anchoring Cdc14 in the nucleolus.

To determine whether sequestration of Cdc14 by Cfi1 in the nucleolus is important in regulating the activity of Cdc14, we examined the phenotype of cells deleted for *CFI1*. Such cells were severely growth-retarded at 25 °C and even more so at 37 °C (data not shown). These *cfi1Δ* cells showed a 15-min delay in bud formation (Fig. 4c) and then formed elongated buds (data not shown). DNA replication and entry into mitosis, as judged by mitotic spindle formation, was more delayed (30 min; Fig. 4d, e).

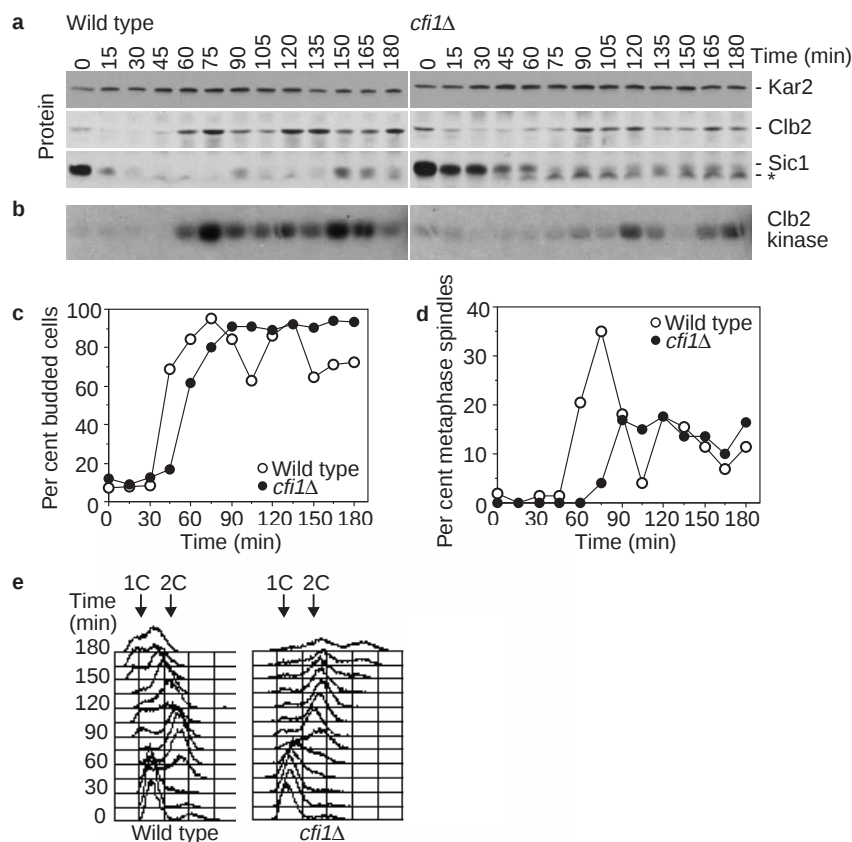


Figure 4 Cells lacking *CFI1* are impaired in activation of mitotic kinases. **a**, Clb2, Sic1 and Kar2 protein; **b**, Clb2-associated kinase activity; **c**, Percentage of budded cells; **d**, Percentage of cells with metaphase spindles; and **e**, DNA content of wild-type (K1534) and *cfi1::URA3* mutant (A1706) cells in synchronous culture after

release from an α -factor-induced G1 arrest. Kar2 protein was used as an internal loading control in immunoblots. Asterisk in **b** indicates Sic1 degradation products. 1C and 2C refer to the number of copies of the genome in the cell.

This phenotype is similar to that caused by overexpression of *CDC14* (ref. 3). However, whereas overexpression of *CDC14* is lethal, deletion of *CFI1* is not.

Overexpression of *CDC14* leads to ectopic inactivation of mitotic kinases, due to hyperaccumulation of Sic1 and inappropriate degradation of Clb cyclins³. Deletion of *CFI1* had a similar but less severe effect. Wild-type and *cfi1*-deletion cells were synchronized in G1 using α -factor pheromone and released into the cell cycle. Whereas Clb2 protein accumulated to high levels 60 min after release from the α -factor block in wild-type cells, it was slow to accumulate in cells lacking *CFI1* (90 min after release) and never accumulated as much as in wild-type cells (Fig. 4a). The CDK inhibitor Sic1 decayed 15 min after release in wild-type cells. In contrast, Sic1 protein was slow to decay in *cfi1Δ* cells (60 min after release; Fig. 4a). This delayed the activation of Clb2-associated kinase, and there was substantially less activity in *cfi1Δ* cells (Fig. 4b). Clb2-associated kinase activity was more strongly affected by deleting *CFI1* than were Clb2 and Sic1 protein levels (Fig. 4b), an observation that is under investigation. Eventually, Clb2 accumulates and Sic1 decays in cells lacking *CFI1* (Fig. 4a). The mechanism(s) that finally override the effects of *CFI1* on Clb2 degradation and Sic1 accumulation, allowing Clb2 to accumulate and Sic1 to decay, are unknown.

In cells arrested in metaphase using nocodazole, there are large amounts of the mitotic cyclin Clb2, and Sic1 protein is absent^{3,24}. In cells lacking *CFI1*, there are low levels of Clb2 and high levels of Sic1 (data not shown). Furthermore, *cfi1Δ* cells escaped the nocodazole arrest and prematurely exited from mitosis, as judged by continuous bud formation (data not shown). Given that the phenotype of cells lacking *CFI1* is similar to that of cells overexpressing *CDC14*, we

suggest that Cfi1 not only sequesters Cdc14 in the nucleolus, but that it also acts as a negative regulator of Cdc14 activity.

If Cfi1 is a negative regulator of Cdc14, then overexpression of *CFI1* should cause the same phenotype as absence of *CDC14* function. Consistent with this, overexpression of *CFI1* from the *GAL1–10* promoter was lethal, causing cells to accumulate in telophase (Fig. 5c, d) with high levels of Clb2 and low levels of Sic1 (Fig. 5a). As a result, Clb2-associated kinase activity was high (Fig. 5b). Thus, overexpression of *CFI1* causes the same phenotype as loss of *CDC14* function. This finding, together with our results showing that loss of *CFI1* mimics a *CDC14*-overproducing phenotype, indicate that Cfi1 is negative regulator of Cdc14. Whether Cfi1 also regulates other proteins is unknown. However, inhibiting Cdc14 is likely to be the critical function of Cfi1, because overexpression of *CDC14* cured the lethality brought about by overexpression of *CFI1* (data not shown). It is still unclear how Cfi1 inhibits Cdc14. As Cdc14–HA is present throughout *GAL–CFI1* cells (Fig. 3f) but these cells exhibit a *CDC14*-loss-of-function phenotype, it is likely that Cfi1 not only inhibits Cdc14 by sequestering it in the nucleolus but also, by inhibiting Cdc14's catalytic activity.

Our results indicate that Cfi1 is a negative regulator of Cdc14. During G1, S phase and early mitosis, Cfi1 binds to and sequesters Cdc14 in the nucleolus, thereby preventing it from dephosphorylating its cell cycle targets. We do not know whether Cfi1 and Cdc14 have functions in the nucleolus. After the start of nuclear division, Cdc14 is released from Cfi1, spreads throughout the nucleus and cytoplasm and triggers exit from mitosis by dephosphorylating Swi5, Sic1 and Cdh1. The release of Cdc14 from Cfi1 during anaphase requires all the components of the mitotic exit pathway.

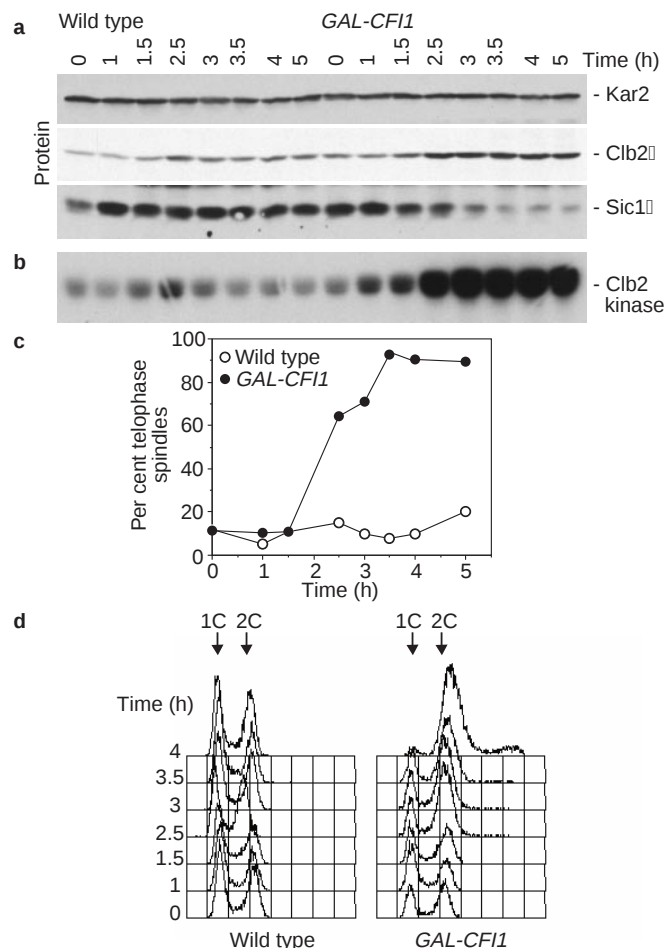


Figure 5 Overexpression of *CFI1* induces high levels of Clb2-associated kinase activity and cell-cycle arrest in telophase. Wild-type (K699) and cells over-expressing *CFI1* (A1662) were grown to exponential phase and treated with galactose to induce Cfi1 accumulation. **a**, Clb2, Sic1 and Kar2 protein. **b**, Clb2-associated kinase activity. **c**, Percentage of cells in telophase. **d**, DNA content.

Table 1 Suppression of various *cdc* mutants by *cfi1*Δ

	Growth at 37°C
<i>tem1-1</i>	-
<i>tem1-1, cfi1</i> Δ	+
<i>cdc5-1</i>	-
<i>cdc5-1, cfi1</i> Δ	+/-
<i>cdc15-2</i>	-
<i>cdc15-2, cfi1</i> Δ	+
<i>dbf2-2</i>	-
<i>dbf2-2, cfi1</i> Δ	+
<i>cdc20-1</i>	-
<i>cdc20-1, cfi1</i> Δ	-

Deletion of *CFI1* rescues the temperature-sensitive growth defect at 37°C of *cdc15-2*, *dbf2-2* and *tem1-1* mutants (+), and partially that of *cdc5-1* mutants (+/-), but not of *cdc20-1* mutants (-).

As this pathway includes at least four protein kinases, phosphorylation may be involved in liberating Cdc14. Promoting the dissociation of Cdc14 from Cfi1 is likely to be the sole essential cell-cycle function of the mitotic exit pathway, as deletion of *CFI1* rescues the temperature-sensitive lethality of *cdc15-2*, *dbf2-2* and *tem1-1* mutants and partially rescues that of *cdc5-1* mutants (Table 1). The mechanisms coupled to nuclear division, or regulators of this process, that trigger the release of Cdc14 from the nucleolus are unknown. Moreover, whether sequestration in the nucleolus is commonly used to inhibit the function of enzymes remains to be determined.

Methods

All strains were derivatives of yeast strain W303 (K699), except the *mccl1-1* mutant (A364a derivative). The *CDC14-HA* construct was integrated as described⁵. *CFI1* was cloned under the control of the *GAL1-10* promoter as described for *CDC14* (ref. 3). *CFI1* was disrupted by replacing the internal *Clal-HpaI* fragment with *URA3*. HA or Myc tags were introduced into *CFI1* as described²⁵. Conditions for growth and release of synchronous cultures from arrest by α -factor were as described¹². Indirect immunofluorescence was done as described^{12,27}. For co-localization studies using primary antibodies raised in rat and mouse (rat anti-tubulin and mouse anti-HA (BABCO), or rat anti-HA (Boehringer) and mouse anti-Myc antibodies (BABCO)), secondary antibodies (Jackson Laboratories) exhibiting minimal cross-reactivity were used. All other techniques were as described in ref. 3 and references therein. Sequence alignments were performed using the Clustal W method using the PAM250 substitution matrix²⁸.

Received 11 February; accepted 22 March 1999.

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Acknowledgements. We thank V. Guacci, L. Guarente, L. Pillus, S. Bell and F. Klein for gifts of strains and antibodies; F. Lewitter for help with sequence alignments; and M. Tyers, S. Bell, T. Orr-Weaver, P. Sorger and S. Prinz for critically reading the manuscript.

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Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex

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Nuclear receptors modulate the transcription of genes in direct response to small lipophilic ligands. Binding to ligands induces conformational changes in the nuclear receptors that enable the receptors to interact with several types of cofactor that are critical for transcription activation (transactivation)¹. We previously described a distinct set of ligand-dependent proteins called DRIPs, which interact with the vitamin D receptor (VDR); together, these proteins constitute a new cofactor complex². DRIPs bind to several nuclear receptors and mediate ligand-dependent enhancement of transcription by VDR and the thyroid-hormone receptor in cell-free transcription assays^{2,3}. Here we report the identities of thirteen DRIPs that constitute this complex, and show that the complex has a central function in hormone-dependent transactivation by VDR on chromatin templates. The DRIPs are almost indistinguishable from components of another new cofactor complex called ARC, which is recruited by other types of transcription activators to mediate transactivation on chromatin-assembled templates^{4,5}. Several DRIP/ARC subunits are also components of other potentially related cofactors, such as

CRSP⁶, NAT⁷, SMCC⁸ and the mouse Mediator⁹, indicating that unique classes of activators may share common sets or subsets of cofactors. The role of nuclear-receptor ligands may, in part, be to recruit such a cofactor complex to the receptor and, in doing so, to enhance transcription of target genes.

The DRIP (for vitamin-D-receptor interacting protein) complex binds nuclear receptors in a completely ligand-dependent manner at the carboxy-terminal α -helical region², called activation function-2 (AF-2; reviewed in ref. 10) of the nuclear receptor's ligand-binding domain (LBD). To establish the identities of individual components of the DRIP complex, we carried out mass-spectrometric and limited microsequence analysis of individual bands resolved by SDS-polyacrylamide gel electrophoresis (PAGE) following depletion of a transcriptionally active nuclear extract prepared from human Namalwa B cells. The DRIPs selectively bound to the VDR LBD immobilized to glutathione-agarose beads only in the presence of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃; Fig. 1a).

Protein-sequencing data showed that each polypeptide analysed from the DRIP complex matched sequences encoded by either mouse or human expressed sequence tags (ESTs), or had identities to polypeptides encoded by known genes (Table 1). DRIP205 is identical to RB18A¹¹ and TRAP220 (ref. 12); the latter protein was originally identified as a subunit of TRAP, a thyroid-hormone-receptor interacting complex that is very similar or identical to the DRIP complex and which was isolated from cells when complexed with epitope-tagged thyroid-hormone receptor in the presence of thyroid hormone³. DRIP205 is also highly homologous to both mouse PBP¹³, a protein that binds peroxisome proliferator-activated receptor- γ , and TRIP2 (ref. 14), encoded by a partial complementary DNA that overlaps the PBP cDNA; each of these proteins was isolated from independent yeast two-hybrid screens. Three other DRIPs, 250, 240 and 100, are encoded by cDNAs that are identical to the partial or full-length cDNAs KIAA0593, KIAA0192 and KIAA0130, respectively, each previously sequenced from a group of 200 clones isolated from human myeloid KG-1 cells¹⁵. We described the full-length sequence and preliminary characterization of DRIP100 previously². Using ESTs identified here, we isolated and sequenced three new full-length cDNAs encoding DRIPs 130, 92 and 77 (polypeptide sequences are given in Supplementary Information). DRIPs 97 and 70-2 correspond to human

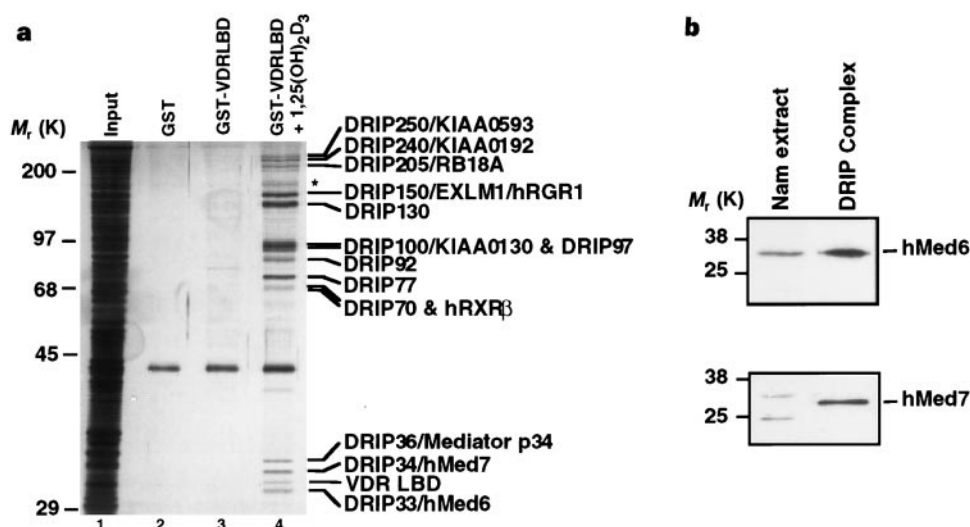


Figure 1 The VDR ligand-binding domain stably interacts with the DRIP complex in a fully hormone-dependent manner. **a**, Immobilized GST-VDR-LBD was incubated with human Namalwa B cell nuclear extract (lane 1) in the absence (ethanol, lane 3) or presence (lane 4) of 1 μ M 1,25(OH)₂D₃. VDR-interacting proteins (DRIPs) were eluted by incubation with Sarkosyl, separated by SDS-PAGE and analysed by silver nitrate staining. Immobilized GST (lane 2) was used as a control protein in the presence of ligand. Each DRIP (and its homologues) is

identified on the right of the gel. The asterisk denotes a 180K protein that is not part of the stable DRIP complex, as determined by glycerol-gradient sedimentation. Note that apparent M_r values of several DRIP subunits have been recalibrated since their original publication². **b**, Med6 and Med7 are components of the DRIP complex. We immunoblotted 10 μ g crude Namalwa nuclear extracts (lane 1) and 10 μ l purified DRIP complex (lane 2) with antibodies against human Med6 or Med7.

Table 1 Identification of DRIP subunits

DRIP	Peptide sequence	Database match(es)	Identity/homologue(s)
250	LGQHRPVS LMLEPYGSQR	GenBank AB011165	KIAA 0593
240	TGSPLDHPIAPSNLPMPEGNSAFTQQVR	GenBank D83783	KIAA 0192
205	FPQIPVSR DRHESVGHGEDFSK DNPAQDFSTLYGSSPLER	GenBank Y13467	hRB18a/mPBP/TRIP2
150	AGNWPGSPQVSGPSPAAR MPGMSPANPSLHSPVPDASHSPR LEILVEDKETGDGR	GenBank AB006651	EXLM1/yRGR-1
130	FITHISK IICNLKPALK YVLEQPYSR	EST W50659 EST AA197696 EST AA386419 EST AA504481	Cloned here GenBank AF105332
100	GLLPYDKDLFEPTQALLR KFINLNEFTYGSEESTKPASVR	GenBank D50920	KIAA 0130
97	GIHFSIVSPR GLVLPVGGGSAPGPLQSK AAPPALLEPLQPTDVSQDPR	EST AA58936	
92	STHCPVSLACAWSCR APTLPGSAATLQLDGLAR	EST AA418511 EST N28978 TIGRTHC178156	Cloned here GenBank AF106934
77	SDVLQDNKWSHLR FQPSLWPWDSVR	EST AA436771 EST H40332	Cloned here GenBank AF105421
70-1	DLTYSCR DGILLATGLHVHR	SwissProt P28702	hRXR- β
70-2	DYTVNLDDGQVAEAGVKPVR	EST Z31208	
36	LGGGLGVAGNSTR EAEOLATVYQAK	EST AA400109 EST R17240	Mouse Mediator p34

and mouse ESTs (Table 1); we have not yet cloned their full-length cDNAs. We also identified DRIP70-1 as human retinoid-X receptor- β (RXR- β), the heterodimeric partner of VDR, and a band of relative molecular mass 26,000 (M_r 26K) as the VDR LBD, which was presumably cleaved from its glutathione-S-transferase (GST) moiety and co-eluted with the complex.

At least four DRIP subunits are homologous to proteins described to be components of Mediator, which can associate with RNA polymerase II (Pol II) through the Pol II large subunit's carboxy-terminal repeat domain (CTD)¹⁶. DRIP36 is nearly identical to mouse p34 (Table 1), a protein that is co-precipitated by antibodies to human Srb7 and is a subunit of mouse Mediator⁹. Two other human homologues of mouse Mediator proteins, Med7 and Med6^{7,9}, corresponding to DRIP34 and DRIP33, respectively, were detected in the DRIP complex by immunoblotting (Fig. 1b). Finally, DRIP150 is identical to EXLM1, the cDNA for which was first cloned in a screen for human genes that escape chromosome-X-specific methylation¹⁷, and is homologous at its amino terminus to yeast Rgr1 (refs 7, 16).

The presence of several Mediator subunits in the DRIP complex indicated that it might co-purify with Pol II. We were in fact able to detect substoichiometric amounts of Pol II's large subunit by immunoblotting when we used the VDR LBD to purify the DRIPs (B.D.L., unpublished results), indicating that Mediator components, perhaps in addition to other DRIP subunits, could function in part by targeting Pol II to promoters.

Although the DRIP complex was first thought to be specific to nuclear hormone receptors, essentially the same complex, called ARC (for activator-recruited cofactor), binds to and is required for transactivation by other transcription factors, such as SREBP-1a, the p65 subunit of NF- κ B, and VP16 (refs 4, 5). The DRIP/ARC complex also shares several subunits with a smaller complex, called CRSP (for cofactor required for Sp1 activation), in a purified transcription system⁶. These results indicate that distinct activators may use common associated subunits for different functions. Although 12 of the 13 DRIP subunits are identical to those in ARC, the latter complex comprises an extra two or three components⁵.

As binding of the DRIP/ARC complex to nuclear receptors is ligand dependent, one or more subunits must interact directly with the receptor as a consequence of ligand binding. To determine which of the DRIP/ARC proteins bind to VDR in the presence of ligand, we transcribed and translated *in vitro* full-length cDNAs

encoding seven DRIPs/ARCs, and precipitated them using GST-VDR-LBD in the presence or absence of 1,25(OH)₂D₃. A single subunit, DRIP/ARC205, bound the VDR LBD in a ligand-dependent manner (Fig. 2). DRIP/ARC205 is identical to TRAP220, which interacts directly with the thyroid-hormone receptor¹². The interaction between VDR and DRIP/ARC205 occurs through one of two LXXLL amino-acid motifs in the central segment of DRIP/ARC205 (data not shown). The LXXLL consensus motif in an appropriate context appears to be critical for interactions between co-activators and nuclear receptors, at least for those within the AF-2 helix¹⁸⁻²². The entire DRIP/ARC complex can be dissociated from VDR with a peptide that recognizes the second LXXLL motif of DRIP/ARC205, indicating that this subunit may anchor the entire complex to the liganded nuclear receptor (data not shown).

We reported previously that transcriptional responsiveness to ligand was lost upon depletion of a DRIP-containing extract with

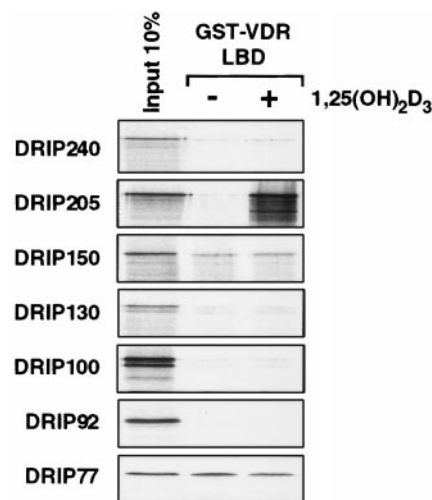


Figure 2 A single DRIP subunit binds directly to VDR in a ligand-dependent manner. Left, *in vitro*-translated, ³⁵S-labelled DRIP/ARC polypeptides were used in each GST pull-down assay; 10% of the input for each translated DRIP protein is shown. Right, GST-VDR-LBD pull-downs were done in the absence and presence of 1 μ M 1,25(OH)₂D₃.

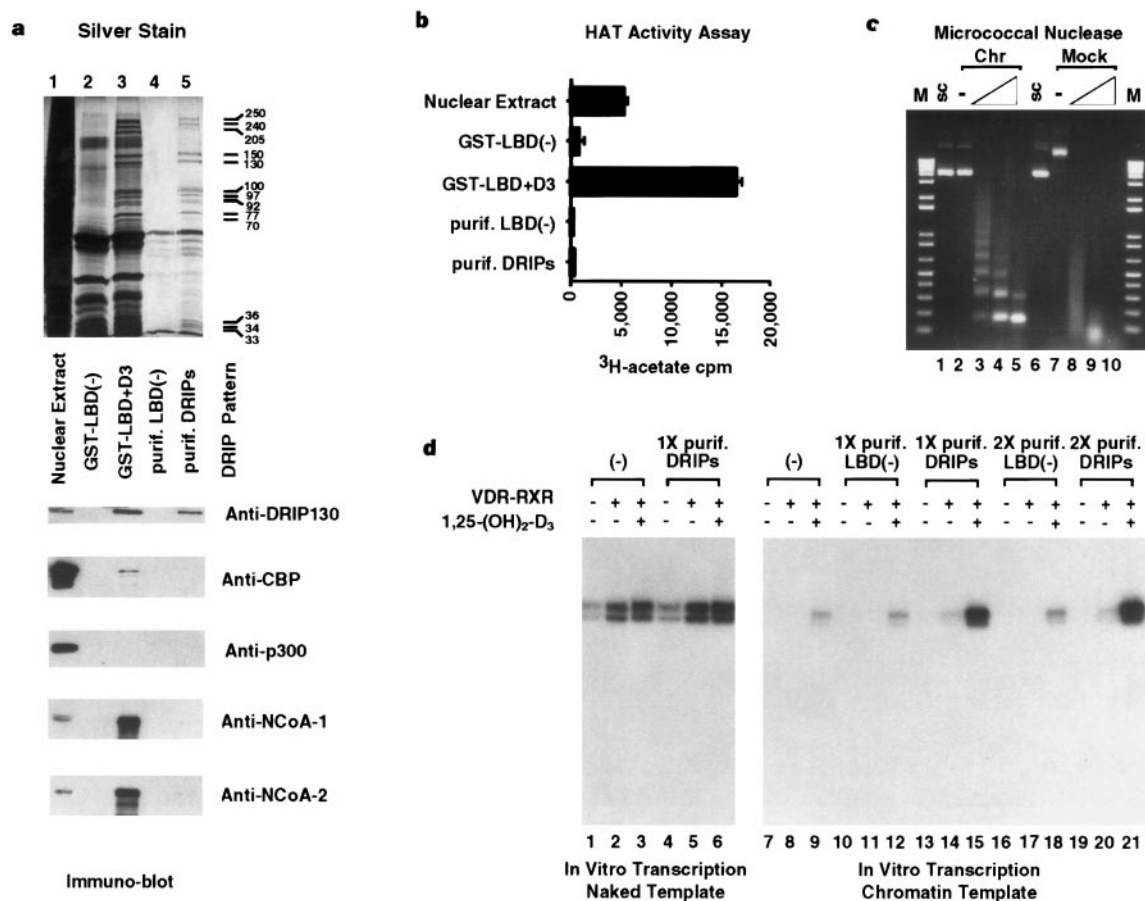


Figure 3 A chromatin template and a highly purified DRIP/ARC complex that lacks HAT activity are required for marked ligand-dependent transcription by VDR-RXR *in vitro*. **a**, Silver stain (top) and immunoblot (bottom) analyses of DRIP-complex fractions isolated from HeLa-cell nuclear extracts resolved on a 7% polyacrylamide-SDS gel. Lane 1, 5 μ l nuclear extract (1:1,000 input); lanes 2, 3, 4 μ l glutathione eluates of bound proteins from GST-VDR-LBD incubated in the absence of ligand (lane 2) or in the presence of 1 μ M 1,25(OH)₂D₃ (lane 3); lanes 4, 5, 10 μ l pooled fractions from glycerol-gradient purification of eluates from lane 2 (lane 4), and from lane 3 (lane 5). The banding pattern of the DRIP complex is depicted at the right of the top gel. Immunoblots of the five fractions shown in the silver-stained gel were performed with antibodies raised against DRIP130, CBP, p300, NCoA-1, and NCoA-2. **b**, Liquid HAT assays were carried out as described² on the five fractions shown in **a**. HAT activity was measured as the amount of ³H-

labelled acetate transferred from ³H-labelled acetyl CoA to histones. **c**, Analysis of digestion by micrococcal nuclease of mock-assembled (lanes 7–10) and chromatin-organized (lanes 2–5) plasmid used as templates for *in vitro* transcription reactions. M, markers; sc, supercoiled plasmid; –, undigested templates; triangles denote increasing concentrations of micrococcal nuclease (see Methods). **d**, Primer-extension analysis of *in vitro* transcription reactions from mock-assembled templates (naked template) and chromatin-organized templates in the absence or presence of nuclear receptors, ligand and purified DRIP complex as indicated (1x and 2x represent 1.5 μ l and 3 μ l purified fractions, respectively). The plasmid template pVDREx4-B/INR-CAT contained four consensus DR3-response elements; primer extension was from the 5' end of the chloramphenicol acetyltransferase (CAT) gene.

immobilized VDR LBD in the presence but not absence of ligand². The thyroid-hormone-receptor/TRAP complex isolated from cells significantly enhanced transcription *in vitro* in both crude extracts and a partially purified transcription system³. In our cell-free transcription assay, however, a component of transcription activation by VDR-RXR was ligand independent, and we proposed that this was probably due to the lack of chromatin assembly in our assay²³. Fully ligand-dependent transcription by the oestrogen receptor can be achieved with chromatin-organized templates *in vitro* in the presence of a crude nuclear extract²⁴. We therefore investigated whether responsiveness to VDR-RXR would become ligand-dependent and, potentially, DRIP/ARC-dependent in a chromatin context.

To study the activity of the DRIP complex in a chromatin context, we assembled chromatin-organized templates in the presence of a crude *Drosophila* cytosolic extract, S190 (Fig. 3c, lanes 2–5); we also assessed transcriptional activity of VDR-RXR at mock-assembled templates (Fig. 3c, lanes 7–10). When we used a highly purified transcription system as the source of basal factors, together with mock-assembled ('naked') DNA templates, we observed only

modest, primarily ligand-independent enhancement of transcription (Fig. 3d, lanes 1–3); the addition of highly purified DRIPs (Fig. 3a, lane 5) enhanced transcription only weakly (Fig. 3d, lanes 4–6). In contrast, we detected moderate but completely ligand-dependent transcription activation by VDR-RXR on chromatin-organized templates (Fig. 3d, lanes 7–9). The purified DRIP complex strongly potentiated ligand-dependent transcription activation by VDR-RXR in a dose-responsive manner (Fig. 3d, lanes 13–15 and 19–21) and transcription was largely unaffected by equivalent amounts of fractions purified using the LBD in the absence of ligand (Fig. 3d, lanes 10–12, 16–18). Moreover, when independently purified preparations of the ARC complex were assayed for VDR-RXR-regulated transcription, >50-fold enhancement by ligand was typically observed (B.D.L. and A.M.N., unpublished results).

The transcription p160 co-activators NCoA-1/SRC1 or NCoA-2/GRIP1/TIF2, which can bind from nuclear extracts to immobilized, liganded VDR, did not co-purify with the transcriptionally active DRIP complex on a glycerol gradient (Fig. 3a: compare lanes 3, 5); in addition, the co-activators CBP and p300 were also absent

from the purified DRIP complex, although they were relatively abundant in the crude nuclear extract (Fig. 3a: compare lanes 1, 3, 5). Moreover, although histone acetyltransferase (HAT) activity was detected in the LBD-bound fraction in the presence of ligand², the gradient-purified DRIP complex lacked HAT activity (Fig. 3b). Thus, although both endogenous p160/CBP co-activators and DRIP complexes bind to nuclear receptors, they appear to do so as functionally distinct complexes. These observations, taken together with the apparent dependence of marked co-activation by DRIP/ARC on chromatin templates, indicate that the DRIP/ARC complex might contain, or interact with, as-yet undefined chromatin-modifying or remodelling activities. Although we do not know what direct function DRIP/ARC might have in a chromatin context, we have for the first time, to our knowledge, shown the importance of this complex in potentiating ligand-dependent transcription from chromatin-organized templates by nuclear receptors, linking them biochemically to their putative functional relevance *in vivo*. The relationship to Mediator, indicates that the DRIP/ARC complex might interact with other Mediator and SRB polypeptides or, alternatively, might itself have similar functions, including, but not limited to, potential direct interaction with and recruitment of Pol II machinery to the promoter.

The DRIP and TRAP complexes were first isolated when bound to nuclear receptors in a ligand-dependent fashion, indicating that the complexes may have been designed to link receptors specifically to the transcriptional apparatus. This view must now be revised with the concurrent discovery of the essentially identical complex (ARC) that interacts with several other classes of transcriptional activators⁴, the characterization of a smaller cofactor complex (CRSP) that contains DRIP/ARC polypeptides and is critical for Sp1-activated transcription⁶, and the description of two highly similar SRB-interacting complexes, NAT⁷ and SMCC⁸, that repress or activate transcription and contain at least three subunits of DRIP/ARC/TRAP. The model of transcriptional regulation by nuclear hormone receptors appears to be converging on the recruitment of co-activator or repressor complexes and subcomplexes composed of shared polypeptides. The selectivity of transcriptional control may lie in subtle differences in the particular composition of these large, multiprotein complexes; selectivity may be influenced by individual or heterotypic combinations of transactivators and the context of enhancer sequences to which the transactivators are bound at the promoters of specific target genes. □

Methods

DRIP-complex isolation. Immobilized GST-VDR-LBD (20 µg) was incubated with 2–4 mg Namalwa nuclear extract as described². Samples were resolved by SDS-PAGE and analysed by silver nitrate staining. Purification of the DRIP/ARCs for functional assays is described below.

Protein sequencing. Samples prepared according to the procedure mentioned above were transferred onto nitrocellulose membranes, stained, digested, and fractionated by reverse-phase HPLC using a 0.8-mm Vydac C18 column²⁵. Peak fractions were analysed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS; Reflex III, Bruker-Franzen, Bremen, Germany) and automated Edman sequencing (477A, Applied Biosystems). In some cases, the peptide mixtures were fractionated on a microtip²⁶ and resulting peptide pools were analysed by MALDI-TOF MS and by electrospray ionization mass spectrometry (ESI MS) on an API 300 triple-quadrupole instrument (PE-SCIEX, Thornhill, Canada) modified with a JAFIS source²⁷. Selected mass values from the MALDI-TOF experiments were taken to search a protein database (NRDB EBI, Hinxton, UK) using the PeptideSearch algorithm. Spectra from the ESI MS/mass-spectrometry analyses were inspected for yⁿ ion series and the resulting information was transferred to the SequenceTag²⁸ program and used as a search string.

Cloning of DRIP/ARC 77, 92 and 130 cDNAs. Human EST clones (Table 1) were obtained from ATCC, labelled by random priming and used to screen a human B-cell library (ZAP-Express, Stratagene). Full-length cDNAs encoding DRIP/ARCs 77, 92 and 130 were confirmed by DNA sequencing, tagged with a

Flag epitope (Kodak) and subcloned into pcDNA3 (Invitrogen) for *in vitro* transcription/translation in rabbit reticulocyte lysates.

GST-affinity-binding assays. Binding assays were done with labelled proteins synthesized *in vitro* using the TnT coupled reticulocyte-lysate system (Promega) in the presence of [³⁵S]methionine (Amersham). We incubated 2 µg immobilized GST-VDR-LBD as described above for 2 h with 2–4 µl labelled proteins. Samples were resolved by SDS-PAGE and analysed by autoradiography.

Antibodies and immunoblot assays. A peptide matching amino acids 892–911 in DRIP130 was synthesized (Research Genetics), conjugated to keyhole limpet haemocyanin (Pierce), and used for antibody production in rabbits (Covance). Anti-CBP (A22, N20) and anti-p300 (N15) antibodies were obtained from Santa Cruz Biotechnologies. Antisera were diluted 1:1,000 (except anti-NCoA1 and anti-NCoA2 antisera, 1:2,000) for western blot analysis which was done using standard methods and visualized by enhanced chemiluminescence (Amersham).

Chromatin assembly. Supercoiled pVDREx4-B/INR-CAT template (4.8 µg ml⁻¹) was incubated at 27 °C for 5 h with *Drosophila* embryo S190 extracts (2 mg ml⁻¹) and highly purified *Drosophila* embryo core histones (~15 µg ml⁻¹) in the presence of 8 mM MgCl₂, 4 mM ATP and an ATP-regenerating system nearly as described²⁹. Chromatin (60 ng plasmid) was then immediately used in transcription reactions *in vitro* as described below, or was digested with 0.03, 0.15 or 0.75 units per ml micrococcal nuclease (Sigma) for 10 min at 22 °C, RNase-A-treated, deproteinized and resolved on agarose gels and visualized with ethidium bromide.

DRIP-complex isolation for transcription assays. Proteins bound to immobilized GST-VDR-LBD (50 µg ml⁻¹) were purified from HeLa cells in the absence or presence of 1 µM 1,25(OH)₂D₃ as described², except that the buffer used was HEGN (20 mM HEPES, pH 7.6, 0.1 mM EDTA, 10% glycerol, 0.1% N-P40, 1 mM dithiothreitol, 1 mM sodium metabisulphite, 0.5 mM phenylmethylsulphoxide, 1 mM benzamidine) plus 0.15 M KCl and 0.5 mg ml⁻¹ BSA. Bound proteins were eluted with 20 mM reduced glutathione in HEGN containing 0.025% NP40 and 0.1 M KCl. 200-µl eluates (~31 HeLa cell equivalent) were sedimented on a 2-ml 15–40% glycerol gradient at 50,000 r.p.m. (Beckman TLS 55 rotor) for 7 h. DRIP-complex-containing fractions assessed by silver staining were used for immunoblotting, HAT assays², and transcription assays. We estimated that there was ~1 µg DRIP130 per 5 × 10⁹ cells and that we purified the DRIP complex ~10,000-fold from nuclear extract.

In vitro transcription. Recombinant Flag-hRXRα and hVDR were over-expressed and purified as described²³. RXR and VDR were preincubated together at final concentrations of 10 nM and 30 nM, respectively, in the presence or absence of 1 × 10⁻⁷ M, 1,25-(OH)₂D₃, and left on ice for 1 h. Transcription assays were done as described⁴, with the following modifications: chromatin or mock-assembled 'naked' templates were added to receptors and incubated for 45 min, at which time the transcription system was added and preincubated for 10 min. NTPs were then added to 0.5 mM and transcription was allowed to proceed for 30 min in final transcription conditions using highly purified human basal transcription factors and conventionally purified TFIID (15 ng), immunopurified TFIID (25 ng), and a cofactor fraction (100 ng), each of which have been described⁴. The latter fraction was further immunodepleted with anti-ARC105/TIG1 rabbit polyclonal antisera as described⁵.

Received 20 January; accepted 18 February 1999.

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Supplementary information is available on Nature's World-Wide Web site or as paper copy from the London editorial office of Nature.

Acknowledgements. We thank L. Lacomis, M. Lui, A. Grewal and S. Geromanos for help with sequencing and mass-spectrometric analysis; M. Mann for the PeptideSearch and SequenceTag programs; J. Lee for the B-cell cDNA library; D. Reinberg and H. Cho for human Med6 antibody; R. Kornberg for human Med7 antibody; C. Glass and M. G. Rosenfeld for NCoA-1 and NCoA-2 antibodies; H. Yoshikawa for EXLM1 cDNA; T. Nagase and the Kazusa DNA Research Institute for KIAA 0593, 0192, and 0130 cDNAs; C.-P. Chang and K. Bark for technical assistance; M. Haggart for DNA sequencing; and C. Inouye for purified recombinant human basal factors TFIIA, -E, and -F. This work was supported by grants from the NIH and the Human Frontiers Science Program (to L.P.F.); B.D.L. is a recipient of a National Research Service Award. Z.S. was supported by the Robert Wood Johnson Jr Charitable Trust.

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Composite co-activator ARC mediates chromatin-directed transcriptional activation

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Gene activation in eukaryotes is regulated by complex mechanisms in which the recruitment and assembly of the transcriptional machinery is directed by gene- and cell-type-specific DNA-binding proteins¹. When DNA is packaged into chromatin, the regulation of gene activation requires new classes of chromatin-targeting activity². In humans, a multisubunit cofactor functions in a

chromatin-selective manner to potentiate synergistic gene activation by the transcriptional activators SREBP-1a and Sp1 (ref. 3). Here we show that this activator-recruited cofactor (ARC) interacts directly with several different activators, including SREBP-1a, VP16 and the p65 subunit of NF-κB, and strongly enhances transcription directed by these activators *in vitro* with chromatin-assembled DNA templates. The ARC complex consists of 16 or more subunits; some of these are novel gene products, whereas others are present in other multisubunit cofactors, such as CRSP⁴, NAT⁵ and mammalian Mediator⁶. Detailed analysis indicates that the ARC complex is probably identical to the nuclear hormone-receptor cofactor DRIP⁷. Thus, ARC/DRIP is a large composite co-activator that belongs to a family of related cofactors and is targeted by different classes of activator to mediate transcriptional stimulation.

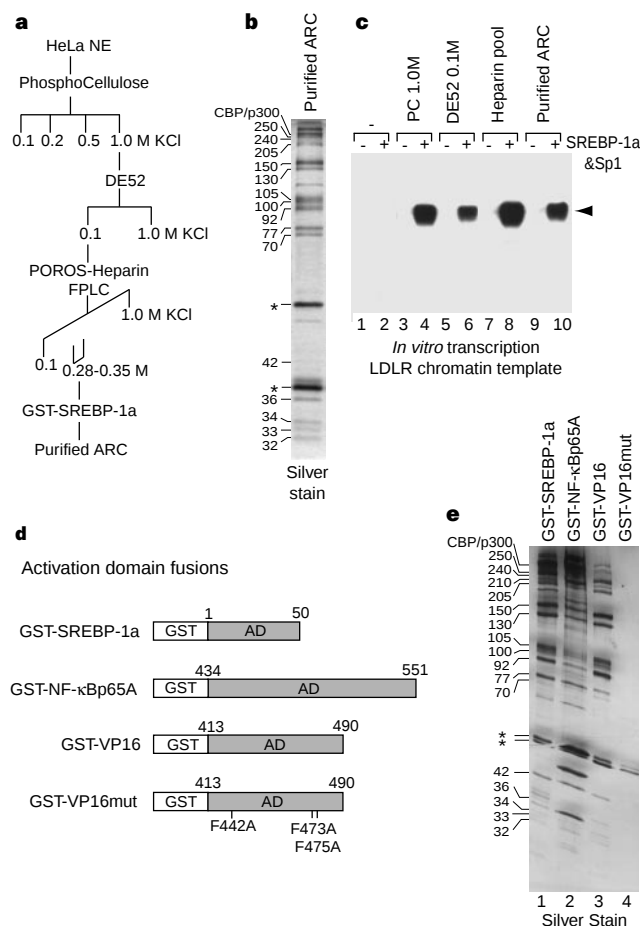


Figure 1 Purification of ARC. **a**, Outline of purification strategy used to initially define the polypeptides composing ARC. **b**, SDS-PAGE and silver-staining analysis of purified ARC complex. The relative molecular masses of polypeptides co-purifying with ARC activity are shown on the left. Stars denote contaminating proteins. **c**, ARC purification was monitored by *in vitro* transcription with SREBP-1a/Sp1-activated transcription at the LDLR-derived chromatin template. Transcription reactions were performed in the presence or absence of SREBP-1a (5 nM) and Sp1 (2 nM), together with cofactor-containing fractions and LDLR chromatin template. **d**, Schematic depiction of GST fusion proteins containing amino acids 1–50 of SREBP-1a, 434–551 of NF-κB p65 subunit, 413–490 of the HSV1 VP16 protein, and 413–490 of VP16 containing three point mutations (F442A, F473A, and F475A). **e**, The human cofactor complex interacts selectively with three types of activation domain. HeLa-cell nuclear extract proteins binding to the activation domains of SREBP-1a (lane 1), p65 (lane 2), VP16 (lane 3) and VP16 with point mutations (lane 4) were analysed by SDS-PAGE followed by silver staining. Asterisks denote nonspecific proteins binding to glutathione-agarose.

The cooperative activation of the LDL receptor (LDLR) gene *in vitro* by the cholesterol-regulated transcription factor SREBP-1a and Sp1, in addition to TFIID, also requires a novel chromatin-selective cofactor containing CBP/p300 and several additional polypeptides that bind to the activation domain of SREBP-1a³. Here we describe the subunit composition and further functionally characterize this multisubunit cofactor.

Conventional purification of the cofactor by phosphocellulose, DE52 and POROS-heparin chromatography, followed by affinity-purification using the SREBP-1a activation domain, indicated that the cofactor activity purifies as a large complex with a relative molecular mass of 2,000,000 (M_r 2,000K), shown by glycerol-gradient sedimentation and S-500 gel filtration (Fig. 1a–c, and data not shown). We tested whether this large activator complex can associate with other types of activator and potentiate their transcriptional function *in vitro*. We incubated HeLa-cell nuclear extract with immobilized activation domains of SREBP-1a, VP16 and the NF- κ B p65 subunit, fused to the glutathione S-transferase (GST) protein, to test for binding to the cofactor (Fig. 1d). A set of 16–17 polypeptides with M_r ranging from 250K–28K interacted consistently with the activation domains of all three activators (Fig. 1e). This indicates that the multisubunit cofactor is indeed targeted by different types of transcriptional activator. We estimate that this affinity purification results in ~50,000-fold purification of the cofactor complex. The activation domains of both SREBP-1a and NF- κ B p65 target free CBP/p300 directly^{8–10}, which probably explains the large amount of CBP/p300 co-purifying with the cofactor complex when these activation domains are used for affinity purification. In contrast, the VP16 activation domain, which does not directly contact CBP/p300, retains much less CBP/p300, which is potentially recruited through the cofactor complex. To investigate the specificity of cofactor binding to the VP16 activation domain, we also tested a fusion protein containing three point mutations that severely compromise the transcriptional activity of VP16 *in vivo*¹¹ (Fig. 1d). No significant binding of the cofactor complex to the mutated VP16 activation domain is observed (Fig. 1e, lane 4). Given its selective interaction with different classes of activator, we called this complex ARC (for activator-recruited cofactor (ARC)).

We then determined the ability of the ARC complex to stimulate transcriptional activation by different activators on chromatin and naked DNA templates *in vitro*. ARC complex purified by SREBP-1a affinity resin was added to *in vitro* transcription reactions containing three different chromatin-assembled DNA templates reconstituted with purified and recombinant basal factors (Fig. 2). As expected, SREBP-1a/Sp1-dependent synergistic activation of the LDLR-derived template was strongly enhanced by the addition of ARC (Fig. 2a, lanes 1–8). Likewise, ARC greatly stimulated NF- κ B and Sp1-directed activation of human immunodeficiency virus long terminal repeat (HIV LTR) transcription with chromatin-assembled template, while maintaining the synergistic response to these activators (Fig. 2b). Furthermore, transcriptional activation by Gal4-VP16 and Sp1 at a chromatin-assembled Gal4/Sp1-responsive promoter was potentiated by the addition of ARC (Fig. 2c). In a reciprocal experiment, ARC complex affinity-purified using the VP16 activation domain supported activation by SREBP-1a/Sp1 at the LDLR promoter (Fig. 2a, lanes 9–12). Sp1 activation was also enhanced by ARC in the context of the HIV LTR and Sp1/Gal4/Sp1 chromatin templates, although we have been unable to demonstrate a direct interaction between Sp1 and the ARC complex. ARC also affects basal transcription; however, the basal stimulation is typically much less (2–5-fold) than enhancement of activated transcription (10–50-fold). The varying response to ARC may reflect differences in activator/promoter sensitivity to ARC, which will be explored in future studies. When testing the ability of ARC to potentiate activation on naked DNA templates by these different activators, less than twofold stimulation was observed with a highly purified *in vitro* transcription system, similar to our previous findings with SREBP-1a and Sp1 at the LDLR DNA template³ (data not shown). These results are consistent with the ARC complex functioning as a transcriptional co-activator for different classes of activator in the context of chromatin.

To characterize the subunit composition of the ARC complex, we affinity-purified ARC from HeLa-cell nuclear extract using the SREBP-1a and VP16 activation domains. At least 16 distinct polypeptides, with M_r between 250K and 28K, consistently co-purified with the cofactor activity. Multiple peptide sequences derived from individual ARC subunits revealed that several are

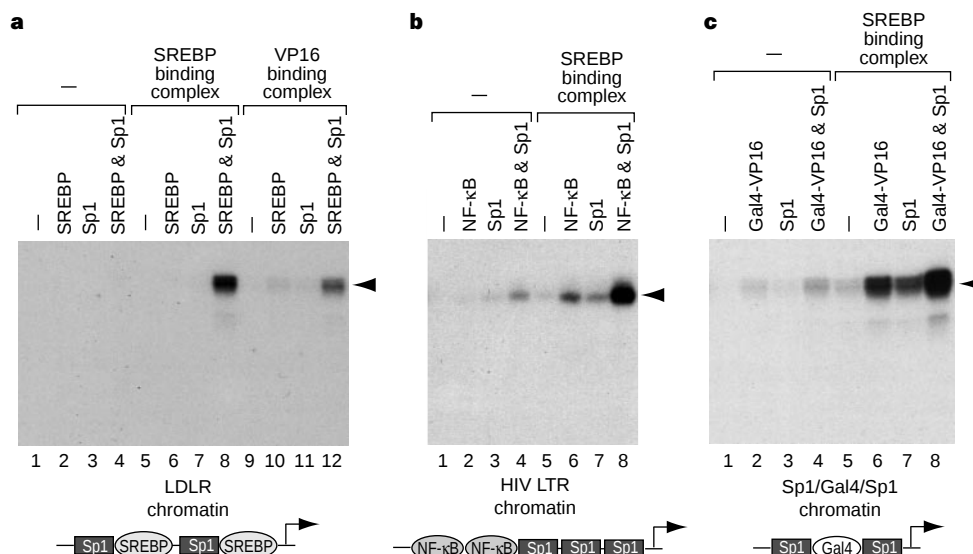


Figure 2 ARC potentiates chromatin-dependent transcriptional activation by several different activators. **a**, ARC complex purified using the SREBP-1a (lanes 5–8) and VP16 (lanes 9–12) activation domains was added to transcription reactions with or without SREBP-1a/Sp1 and the LDLR promoter-derived chromatin template. **b**, NF- κ B/Sp1-dependent activation of the HIV LTR is enhanced by ARC. SREBP-1a-purified ARC complex was added in lanes 5–8 in

the presence or absence of NF- κ B p65 homodimers and Sp1. **c**, ARC potentiates activation by Gal4-VP16 (Gal4 amino-acids 1–94 fused to VP16 amino-acids 413–490) and Sp1 at a chromatin template harbouring Sp1 and Gal4 binding sites. The organization of activator binding sites in the promoters is shown below each set of transcription results.

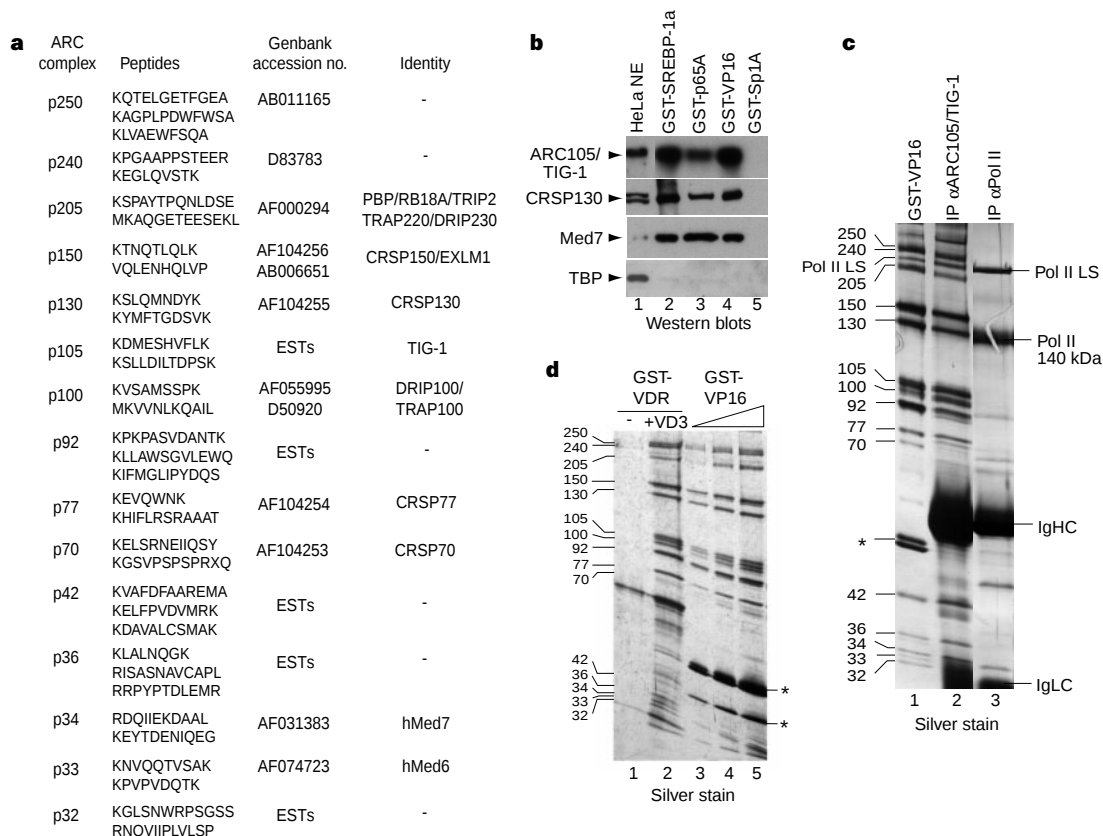


Figure 3 Composition of ARC and identity of subunits. **a**, A comprehensive analysis of ARC subunits separated by their apparent relative molecular mass. Peptide sequences were obtained by microsequencing and used to search the Genbank database. The Genbank accession numbers corresponding to matching entries or a referral to ESTs (see Supplementary Information) are listed together with known identities. **b**, Immunoblotting analysis of ARC complex affinity-purified using the SREBP-1a (lane 2), NF- κ B p65 (lane 3) or VP16 (lane 4) activation domains using antisera directed against ARC105/TIG-1, CRSP 130,

human Med7 and TBP. The Sp1 activation domain A fused to GST (lane 5) was used as a control. **c**, An antibody against ARC105/TIG-1 can immunoprecipitate the ARC complex from HeLa-cell nuclear extract. ARC affinity-purified by GST-VP16 (lane 1) was compared with immunoprecipitations using antibodies against ARC105/TIG-1 (lane 2) and the large subunit of RNA Pol II (lane 3) from HeLa-cell nuclear extract (NE). **d**, ARC and DRIP are probably identical. Silver staining comparing the polypeptide patterns of the DRIP (lane 2) and ARC (lanes 3–5) complexes.

encoded by novel genes with unknown functions, represented by partial complementary DNAs and expressed sequence tags deposited in the Genbank database (for accession numbers, see Supplementary Information) (Fig. 3a). One gene, encoding ARC105, is identical to TIG-1, recently isolated based on induced expression by treatment of K562 cells with 12-*o*-tetradecanoylphorbol-13-acetate (TPA) (S.A. and W.S., manuscript in preparation). Immunoblotting using antiserum raised against ARC105/TIG-1 confirmed its presence in the ARC complex, while TATA-binding protein (TBP) was not detected in any ARC preparation (Fig. 3b). The antibody against ARC105/TIG-1 also immunoprecipitated the ARC complex from HeLa-cell nuclear extract, showing that ARC is a stable complex (Fig. 3c: compare lanes 1 and 2). An antibody against RNA polymerase II (Pol II) failed to precipitate the ARC complex (Fig. 3c, lane 3).

Surprisingly, analysis of the amino-acid sequences derived from individual ARC subunits revealed that a number of the ARC components are shared with other complexes involved in transcriptional regulation. For example, ARC205 is identical/homologous to TRIP2/PBP/RB18A/TRAP220 (refs 12–15), and ARC100 is identical to DRIP/TRAP100 (Fig. 3a), both of which are components of the nuclear hormone-receptor DRIP and TRAP co-activator complexes^{15–17}. These large complexes are recruited to vitamin D₃ and thyroid hormone receptors, respectively, in a ligand-dependent fashion to stimulate transcriptional activation *in vitro*. A direct comparison of polypeptides in our ARC complex with the DRIP complex reveals a remarkably similar pattern⁷ (Fig. 3d). Compara-

tive immunoblotting analysis indicates that ARC105/TIG-1 is present in DRIP prepared from HeLa-cell nuclear extract (data not shown). Additionally, direct comparison of amino-acid sequences between ARC and DRIP polypeptides confirmed that the 250K, 240K, 205K, 150K, 130K, 100K, 77K, 70K, 36K, 34K and 33K ARC subunits are indeed identical to DRIPs with the corresponding relative molecular masses⁷ (see Supplementary Information). Moreover, ARC binds to the retinoic acid receptor- α in a ligand-dependent manner (data not shown). ARC is also required for hormone-inducible activity of the vitamin D₃ receptor *in vitro* at chromatin templates, and purified DRIP complex strongly stimulates SREBP-1a/Sp1-dependent synergy at the LDLR chromatin template (B. D. Lemon and A.M.N., unpublished data). We therefore believe that ARC and DRIP are identical or highly related cofactors, referred to as ARC/DRIP. It is possible that the previously reported TRAP complex^{15,17} is also highly similar or identical to the ARC/DRIP complex. However, direct comparison of these complexes will require identification of the TRAP subunits.

Our laboratory has independently isolated a 600K–700K cofactor complex, CRSP, which is required for Sp1 activation on naked DNA templates, and which contains 8–9 polypeptides with M_r of 200K–33K (ref. 4). Most of the CRSP subunits appear to be contained within the ARC/DRIP complex (Fig. 3a). Immunoblotting using affinity-purified antibodies against CRSP150, 130, 77, 70 and 33 confirmed their presence in the ARC/DRIP complex (Fig. 3b, and data not shown). Thus, CRSP may either represent a subcomplex of ARC/DRIP or a distinct activity sharing many subunits with ARC/

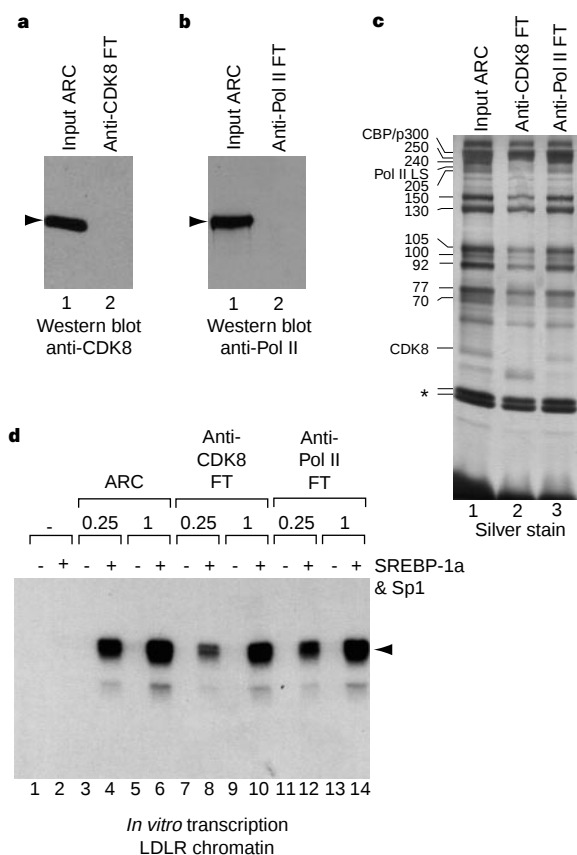


Figure 4 CDK8 and RNA Pol II are not components of ARC. **a**, The immunoblot shows the complete removal by immunodepletion of CDK8 from an ARC preparation. **b**, The large subunit of Pol II is completely removed by immunodepletion from an ARC preparation, as shown in this immunoblot. **c**, The majority of ARC remains after immunodepletion of CDK8 and Pol II. Silver-staining analysis of ARC immunodepleted of contaminating CDK8 and Pol II. **d**, ARC activity is not significantly affected by removal of contaminating CDK8 and Pol II. Transcription reactions were performed in the presence or absence of SREBP-1a (5 nM) and Sp1 (2 nM) with or without affinity-purified ARC (lanes 3–6) or ARC immunodepleted for CDK8 (lanes 7–10) or Pol II (lanes 11–14) and LDLR chromatin template.

DRIP. Future work will address the precise structural and functional relationships between ARC/DRIP and CRSP.

One of the ARC/DRIP and CRSP subunits appears to be a human homologue of the yeast Mediator protein Med7, whereas the 150K subunit shared between these complexes has limited homology to yeast Rgr1 (refs 4, 18) (Fig. 3a, b). In addition, ARC/DRIP (but not CRSP) contains the human Med6 homologue⁵ (Fig. 3a). Two other Mediator protein-containing mammalian complexes have been identified. A complex suggested to represent the mouse Mediator contains proteins with homology to yeast Med6, Med7 and Rgr1¹⁶. This complex also appears to contain a 78K homologue of CRSP77 (identical to ARC/DRIP77), as well as a 34K homologue of ARC/DRIP36. However, the overall subunit composition of the mouse complex, as defined, appears to be distinct from that of ARC/DRIP and CRSP, as a significant number of polypeptides appear to differ between the complexes. Thus far, the mouse complex has not been shown to be recruited by activators or to function as an activator-dependent cofactor.

Another human complex, negative regulator of activated transcription (NAT), was identified based on immunoprecipitation with antibodies directed against the human SRB10 homologue CDK8. It also contains human Med6 and Med7, as well as a protein with some homology to yeast Rgr1 (ref. 5). Because the subunit

composition of NAT appears to be similar to that of ARC/DRIP, we performed comparative western blotting and immunoprecipitation studies using antibodies against human CDK8 as well as SDS-PAGE/silver-staining analysis to investigate the potential relationship between NAT and ARC/DRIP. Immunoblotting revealed that some CDK8 was detectable in affinity-purified ARC/DRIP preparations (Fig. 4a, lane 1). However, CDK8 appears to be present only in substoichiometric amounts relative to ARC/DRIP components. Efficient and complete immunodepletion of CDK8 from purified ARC/DRIP preparations showed that most ARC/DRIP proteins remain in the flowthrough fractions (Fig. 4a: compare lanes 1 and 2; Fig. 4c: compare lanes 1 and 2). Moreover, the CDK8-depleted ARC/DRIP complex is highly active in the SREBP-1a/Sp1-dependent chromatin transcription assay, confirming that CDK8 and its associated subunits are not necessary for ARC/DRIP function (Fig. 4d: compare lanes 1–6 with lanes 7–10). Further evaluation of the structural and functional relationship between ARC/DRIP and NAT awaits identification of the remaining NAT subunits.

A characteristic of the yeast Mediator complex is its ability to associate with Pol II, which is thought to occur through the carboxy-terminal repeat domain (CTD) of the large subunit of Pol II¹⁹. The ARC/DRIP complex contains some Mediator proteins, so we tested for Pol II subunits in our affinity-purified ARC complex. Immunoblotting with a monoclonal antibody selective for the unphosphorylated form of CTD revealed substoichiometric amounts of the large Pol II subunit in our ARC/DRIP preparations (Fig. 4b, lane 1). However, in contrast to the Mediator complex, the ARC/DRIP complex (like the NAT complex⁵) does not directly interact with the CTD of Pol II (data not shown). Next, we removed the small amounts of Pol II from our affinity-purified ARC/DRIP preparation by immunodepletion using the anti-Pol II monoclonal antibody (Fig. 4b, lane 2). As expected, nearly all of the ARC/DRIP proteins remained in the flow-through fraction (Fig. 4c: compare lanes 1 and 3). Moreover, the Pol II-depleted ARC/DRIP retained potent cofactor activity, confirming that the low levels of Pol II contaminating the ARC/DRIP preparations do not significantly contribute to the activity of the cofactor (Fig. 4d: compare lanes 1–6 with lanes 11–14). However, the presence of some Pol II in the affinity-purified ARC/DRIP indicates that the ARC/DRIP complex may function at least in part by recruiting Pol II to the promoter. Future experiments will be designed to evaluate the potential involvement of Pol II in the mechanism of transcriptional enhancement by ARC/DRIP.

Our results indicate that ARC/DRIP is a multisubunit composite cofactor important for chromatin-dependent transcriptional stimulation by a variety of activators. The ARC complex can be recruited to promoters by binding directly to different activation domains. In addition, both ARC and TFIID appear to be required to mediate activation of transcription with chromatin templates *in vitro*³ (P.A.B. and A.M.N., unpublished observations). Several of the ARC subunits appear to be integral components of other distinct transcriptional cofactors functioning in activation or repression. Subunit sharing between cofactor complexes has emerged as an important concept in transcriptional regulation. For instance, TFIID appears to share several TBP-associated factors or highly related proteins with the SAGA/PCAF co-activator complexes^{20,21}. This type of combinatorial mixing and matching of co-activator subunits may have evolved in response to the more diverse repertoire of genetic programmes and signalling pathways characteristic of complex organisms. Future efforts will be directed at characterizing the activities associated with the ARC/DRIP complex in more detail to determine their mode of action and contribution to transcriptional regulation. Such studies should shed light on the functional relationship of ARC/DRIP with a growing number of related cofactors and may reveal new mechanisms for regulating transcription in higher eukaryotic organisms.

Note added in proof: We have become aware that a human complex

containing SRB and MED homologues (SMCC) has recently been described. It exhibits functional characteristics and a subunit composition similar to the NAT complex, but also has three components which are subunits of the TRAP complex²². □

Methods

Generation of GST-activation domain fusions. Sequences corresponding to the activation domains of SREBP-1a, NF- κ B p65 and VP16 were amplified using PCR and inserted in frame with sequences encoding glutathione S-transferase. Fusion proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified over glutathione-coupled resin (Pharmacia).

Binding of HeLa-cell nuclear proteins to GST-activation domains. One millilitre of HeLa nuclear extract prepared as described²³ was incubated for 3 h nutating at 4 °C with 20 μ l of GST-fusion-protein-bound resin pre-equilibrated in 0.1 M KCl HEGN (20 mM HEPES, pH 7.6; 0.1 mM EDTA; 10% glycerol; 0.1% NP-40; 1 mM DTT; 1 mM benzamide; 0.25 mM PMSF; 2 μ g ml⁻¹ aprotinin). The beads were washed seven times with 1 ml of 0.5 M KCl HEGN for GST-SREBP-1a and GST-VP16 or 0.25 M KCl HEGN for GST-p65, followed by one wash with 0.1 M KCl HEGN. Bound proteins were eluted by incubating beads twice with 20 μ l of 0.2% Sarkosyl in 0.1 M KCl HEGN or 10 mM glutathione in 0.1 M KCl HEGN for 2 h nutating at 4 °C. The DRIP complex was purified as described⁷. The eluted material was analysed by SDS-PAGE and silver staining or immunoblotting and *in vitro* transcription.

Conventional purification of ARC. HeLa nuclear extract (200 ml) was applied to a 100 ml PhosphoCellulose column pre-equilibrated with 0.1 M KCl HEG. The column was step-eluted using two column volumes of 0.2 M, 0.5 M, and finally 1.0 M KCl HEG. The 1.0 M fraction was then dialysed to 0.1 M KCl and applied to a 50 ml DE52 column pre-equilibrated in 0.1 M KCl HEG. The column was eluted using 1.0 M KCl HEG. The flow-through fraction contained most of the cofactor activity and was loaded onto a 4 ml POROS-Heparin column (FPLC). Bound proteins were eluted by applying a 20-column volume gradient from 0.1 M–1.0 M KCl in HEG buffer. Fractions containing the peak of activity (eluting between 0.28–0.35 M KCl) were pooled and dialysed against 0.1 M KCl HEG. A portion of the pooled material from the Heparin column was then subjected to affinity chromatography using GST-SREBP-1a beads as described above. The eluted material was analysed using SDS-PAGE/silver staining and *in vitro* transcription with SREBP-1a/Sp1 at the LDLR chromatin template.

Immunoprecipitation and immunoblotting. For the immunoprecipitation studies, 4 μ l of anti-ARC105/TIG-1 antiserum (S.A. and W.S., manuscript in preparation), 50 μ l of anti-CDK8 (C-20, Santa Cruz Biotechnology), or 100 μ l of anti-Pol II mouse hybridoma supernatant (8WG16) were incubated with 15 μ l protein A/G beads (Pharmacia) for 1 h nutating at 4 °C. After extensive washing, 1 ml of HeLa nuclear extract was incubated for 3 h nutating at 4 °C with the antibody-coupled protein A/G beads. The beads were washed seven times with 1 ml of 0.5 M KCl HEGN, and once with 1 ml 0.1 M KCl HEGN. CDK8 and Pol II were immunodepleted from ARC by incubating 100 μ l of glutathione-eluted ARC (affinity-purified using the SREBP or VP16 activation domains) twice with anti-CDK8 or anti-Pol II coupled protein A/G beads for 2 h nutating at 4 °C. Specifically bound material and flowthrough fractions were analysed by SDS-PAGE and silver or immunostaining. Immunoblotting was performed essentially as described³.

Purification of ARC. Large-scale purification of ARC was achieved by incubating 100 ml of HeLa nuclear extract (pre-cleared with GST beads twice for 1 h) with 1 ml of GST-VP16 or GST-SREBP-coupled beads for 4 h nutating at 4 °C. The beads were washed extensively with 0.5 M KCl HEGN, followed by two washes with 0.1 M HEGN. Bound proteins were eluted by incubating twice with 1 ml of 0.2% Sarkosyl in 0.1 M KCl HEGN for 2 h nutating at 4 °C.

Protein microsequencing. Purified and concentrated ARC was separated by SDS-PAGE and transferred to nitrocellulose or PVDF membranes. After Ponceau S staining, the bands corresponding to distinct polypeptides were excised and subjected to Lys-C or trypsin digestion followed by high-performance liquid chromatography separation of peptides. The purified peptides were then analysed by microsequencing and sequences were used to search the Genbank database using the BLAST program.

Chromatin assembly. The template plasmids were assembled into chromatin as described²⁴. Briefly, *Drosophila* embryo cytosolic high-speed supernatant (S-

190) extract, purified core histones, Mg/ATP and an ATP-regenerating system were added to supercoiled DNA template and assembly was performed for 4.5 h at 27 °C.

In vitro transcription. Transcription reactions were performed essentially as described³. Briefly, after 4.5 h of chromatin assembly, SREBP-1a (5 nM), NF- κ B p65 homodimers (5 nM), Gal4-VP16 (10 nM) and/or Sp1 (2 nM) were incubated with or without affinity-purified ARC (0.125–0.5 nM) and chromatin template (0.5 nM) for 30 min at 27 °C. For control transcription on naked DNA templates, activators and ARC were added to identical amounts of template (0.5 nM) in the presence or absence of S-190 extract (30 μ g) and Mg-ATP for 30 min at 27 °C. Purified GTFs were added either with or without cofactor fraction³ depleted of ARC by GST-SREBP-1a or an antibody against ARC105/TIG-1 (the ARC-depleted cofactor fraction is not required for transcriptional activation at chromatin templates, but reduces basal levels of transcription) and allowed to incubate for 10 min at 27 °C. Transcription was initiated by addition of NTPs (0.5 mM final concentration) in a final volume of 50 μ l and allowed to proceed for 30 min at 27 °C. The products were analysed by primer extension²⁴.

Received 21 January; accepted 19 March 1999.

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Acknowledgements. We thank P. Kaufman, J. Kadonaga, B. Lemon, J. Zwicker, S. Ryu, A. Ladurner and M. Rabenstein for critical reading of the manuscript; R. Burgess for providing the 8WG16 cell line; Z. Cao for the NF- κ B p65 subunit; B. Lemon for the purified core Pol II and DRIPs; W. Dynan for the plasmid encoding GST-CTD; S. Triezenberg for plasmids containing VP16 point mutations; R. Kornberg for the antibody against human Med7; S. Ryu for antibodies against CRSP subunits; and A. Ladurner for helping with sequence comparisons. This work was funded by grants from the NIH and HHMI.

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